

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/00, C12P 21/06 C07K 3/00, 7/00, 13/00	A1	(11) International Publication Number: WO 92/22646 (43) International Publication Date: 23 December 1992 (23.12.92)
(21) International Application Number: PCT/US92/05000 (22) International Filing Date: 15 June 1992 (15.06.92) (30) Priority data: 717,774 14 June 1991 (14.06.91) US (71) Applicant: DNX CORP. [US/US]; 303B College Road East, Princeton Forrestal Center, Princeton, NJ 08450 (US). (72) Inventors: LOGAN, John, S. ; 347B Old York Road, Robinsville, NJ 08691 (US). HOLTZMAN, Steven ; 15 Sherbrooke Drive, Princeton Junction, NJ 08550 (US). O'DONNELL, J., Kevin ; 2822 Hearth Place, Doylestown, PA 19801 (US). PILDER, Stephen, H. ; 63-10 Ravens's Crest Drive, Plainsboro, NJ 08536 (US). PINKERT, Carl, A. ; 1998 Lakemont Drive, Bessemer, AL 35023 (US). SWANSON, Mark, E. ; 14 Lake Shore Drive, Lake Hiawatha, NJ 07034 (US). KELLER, Hillary ; 3613 Quail Ridge Drive, Plainsboro, NJ 08536 (US).		(74) Agent: MISROCK, S., Leslie; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US). (81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KR, LK, LU (European patent), MC (European patent), MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL (European patent), NO, PL, RO, RU, SD, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent). Published <i>With international search report.</i>
(54) Title: PRODUCTION OF HUMAN HEMOGLOBIN IN TRANSGENIC PIGS (57) Abstract <p>The present invention relates to the use of transgenic pigs for the production of human hemoglobin. The transgenic pigs of the invention may be used as an efficient and economical source of cell-free human hemoglobin that may be used for transfusions and other medical applications in humans.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

PRODUCTION OF HUMAN HEMOGLOBIN IN TRANSGENIC PIGS

5

1. INTRODUCTION

The present invention relates to the use of transgenic pigs for the production of human hemoglobin. The transgenic pigs of the invention may be used as an efficient and economical source of cell-free human hemoglobin that may be used for transfusions and other medical applications in humans.

2. BACKGROUND OF THE INVENTION2.1. HEMOGLOBIN

Oxygen absorbed through the lungs is carried by hemoglobin in red blood cells for delivery to tissues throughout the body. At high oxygen tensions, such as those found in the proximity of the lungs, oxygen binds to hemoglobin, but is released in areas of low oxygen tension, where it is needed.

Each hemoglobin molecule consists of two alpha globin and two beta globin subunits. Each subunit, in turn, is noncovalently associated with an iron-containing heme group capable of carrying an oxygen molecule. Thus, each hemoglobin tetramer is capable of binding four molecules of oxygen. The subunits work together in switching between two conformational states to facilitate uptake and release of oxygen at the lungs and tissues, respectively. This effect is commonly referred to as heme-heme interaction or cooperativity.

The hemoglobins of many animals are able to interact with biologic effector molecules that can further enhance oxygen binding and release. This enhancement is manifested in changes which affect the allosteric equilibrium between the two conformational

states of hemoglobin. For example, human and pig hemoglobin can bind 2, 3 diphosphoglycerate (2,3 DPG), which influences the equilibrium between the two
5 conformational states of the tetramer and has the net effect of lowering the overall affinity for oxygen at the tissue level. As a result, 2,3-DPG increases the efficiency of oxygen delivery to the tissues.

10 2.2. GLOBIN GENE EXPRESSION

Hemoglobin protein is expressed in a tissue specific manner in red blood cells where it accounts for approximately ninety percent of total cellular protein. Thus, red blood cells, which have lost their
15 nucleus and all but a minimal number of organelles, are effectively membrane-enclosed packets of hemoglobin dedicated to oxygen transfer.

Humans and various other species produce different types of hemoglobin during embryonic, fetal,
20 and adult developmental periods. Therefore, the factors that influence globin gene expression must be able to achieve tissue specific control, quantitative control, and developmentally regulated control of globin expression.

Human globin genes are found in clusters on
25 chromosome 16 for alpha (α) globin and chromosome 11 for beta (β) globin. The human beta globin gene cluster consists of about 50 kb of DNA that includes one embryonic gene encoding epsilon (ϵ) globin, two
30 fetal genes encoding gamma (γ) G and gamma A globin, and two adult genes encoding delta (δ) and beta (β) globin, in that order (Fritsch^{et al.}, 1980, Cell 19:959-972).

It has been found that DNA sequences both
35 upstream and downstream of the β globin translation initiation site are involved in the regulation of β

globin gene expression (Wright et al., 1984, Cell 38:263). In particular, a series of four Dnase I super hypersensitive sites (now referred to as the locus control region, or LCR) located about 50 kilobases upstream of the human beta globin gene are extremely important in eliciting properly regulated beta globin-locus expression (Tuan et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 83:1359-1363; PCT Patent Application WO 8901517 by Grosveld; Behringer et al., 1989, Science 245:971-973; Enver et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:7033-7037; Hanscombe et al., 1989, Genes Dev. 3:1572-1581; Van Assendelft et al., 1989, Cell 56:967-977; Grosveld et al., 1987, Cell 51:975-985).

2.3. THE NEED FOR A BLOOD SUBSTITUTE

Recently, the molecular aspects of globin gene expression have met with even greater interest as researchers have attempted to use genetic engineering to produce a synthetic blood that would avoid the pitfalls of donor generated blood. In 1988, between 12 million and 14 million units of blood were used in the United States alone (Andrews, February 18, 1990, New York Times), an enormous volume precariously dependent on volunteer blood donations. About 5 percent of donated blood is infected by hepatitis virus (Id.) and, although screening procedures for HIV infection are generally effective, the prospect of contracting transfusion related A.I.D.S. remains a much feared possibility. Furthermore, transfused blood must be compatible with the blood type of the transfusion recipient; the donated blood supply may be unable to provide transfusions to individuals with rare blood types. In contrast, hemoglobin produced by genetic engineering would not require blood type

matching, would be virus-free, and would be available in potentially unlimited amounts. Several research groups have explored the possibility of expressing hemoglobin in microorganisms. For example, see International Application No. PCT/US88/01534 by Hoffman and Nagai, which presents, in working examples, production of human globin protein in E. coli.

10

2.4. TRANSGENIC ANIMALS

A transgenic animal is a non-human animal containing at least one foreign gene, called a transgene, in its genetic material. Preferably, the transgene is contained in the animal's germ line such that it can be transmitted to the animal's offspring. A number of techniques may be used to introduce the transgene into an animal's genetic material, including, but not limited to, microinjection of the transgene into pronuclei of fertilized eggs and manipulation of embryonic stem cells (U.S. Patent No. 4,873,191 by Wagner and Hoppe; Palmiter and Brinster, 1986, Ann. Rev. Genet. 20:465-499; French Patent Application 2593827 published August 7, 1987). Transgenic animals may carry the transgene in all their cells or may be genetically mosaic.

Although the majority of studies have involved transgenic mice, other species of transgenic animal have also been produced, such as rabbits, sheep, pigs (Hammer et al., 1985, Nature 315:680-683) and chickens (Salter et al., 1987, Virology 157:236-240). Transgenic animals are currently being developed to serve as bioreactors for the production of useful pharmaceutical compounds (Van Brunt, 1988, Bio/Technology 6:1149-1154; Wilmut et al., 1988, New Scientist (July 7 issue) pp. 56-59).

Methods of expressing recombinant protein via transgenic livestock have an important theoretical advantage over protein production in recombinant bacteria and yeast; namely, the ability to produce large, complex proteins in which post-translational modifications, including glycosylation, phosphorylation, subunit assembly, etc. are critical for the activity of the molecule.

In practice, however, the creation of transgenic livestock has proved problematic. Not only is it technically difficult to produce transgenic embryos, but mature transgenic animals that produce significant quantities of recombinant protein may prove inviable. In pigs in particular, the experience has been that pigs carrying a growth hormone encoding transgene (the only transgene introduced into pigs prior to the present invention) suffered from a number of health problems, including severe arthritis, lack of coordination in their rear legs, susceptibility to stress, anoestrus in gilts and lack of libido in boars (Wilmut et al., supra). This is in contrast to transgenic mice carrying a growth hormone transgene, which appeared to be healthy (Palmiter et al., 1982, Nature 300:611-615). Thus, prior to the present invention, healthy transgenic pigs (which efficiently express their transgene(s)) had not been produced.

2.5. EXPRESSION OF GLOBIN GENES IN TRANSGENIC ANIMALS

Transgenic mice carrying human globin transgenes have been used in studying the molecular biology of globin gene expression. A hybrid mouse/human adult beta globin gene was described by Magram et al. in 1985 (Nature 315:338-340). Kollias et al. then reported regulated expression of human gamma-A, beta, and hybrid beta/gamma globin genes in

transgenic mice (1986, Cell 46:89-94). Transgenic mice expressing human fetal gamma globin were studied by Enver et al. (1989, Proc. Natl. Acad. Sci. U.S.A. 86:7033-7037) and Constantoulakis et al. (1991, Blood 77:1326-1333). Autonomous developmental control of human embryonic globin⁺ gene switching in transgenic mice was observed by Raich et al. (1990, Science 250:1147-1149).

10 Transgenic mouse models for a variety of disorders of hemoglobin or hemoglobin expression have been developed, including sickle cell disease (Rubin et al., 1988, Am. J. Human Genet. 42:585-591; Greaves et al., 1990, Nature 343:183-185; Ryan et al., 1990, Science 247:566-568; Rubin et al., 1991, J. Clin. Invest. 87:639-647); thalassemia (Anderson et al., 1985, Ann. New York Acad. Sci. (USA) 445:445-451; Sorenson et al., 1990, Blood 75:1333-1336); and hereditary persistence of fetal hemoglobin (Tanaka et al., 1990, Ann. New York Acad. Sci. (USA) 612:167-178).

Concurrent expression of human alpha and beta globin has led to the production of human hemoglobin in transgenic mice (Behringer et al., 1989, Science 245:971-973; Townes et al., 1989, Prog. Clin. Biol. Res. 316A:47-61; Hanscombe et al., 1989, Genes Dev. 3:1572-1581). It was observed by Hanscombe et al. (supra) that transgenic fetuses with high copy numbers of a transgene encoding alpha but not beta globin exhibited severe anemia and died prior to birth. Using a construct with both human alpha and beta globin genes under the control of the beta globin LCR, live mice with low copy numbers were obtained (Id.). Metabolic labeling experiments showed balanced mouse globin synthesis, but imbalanced human globin

synthesis, with an alpha/beta biosynthetic ratio of about 0.6 (Id.).

5 3. SUMMARY OF THE INVENTION

The present invention relates to the use of transgenic pigs for the production of human hemoglobin and/or human globin. It is based, at least in part, on the discovery that transgenic pigs may be generated
10 that express human hemoglobin in their erythrocytes and are healthy, suffering no deleterious effects as a result of heterologous hemoglobin production.

In particular embodiments, the present invention provides for transgenic pigs that express
15 human globin genes. Such animals may be used as a particularly efficient and economical source of human hemoglobin, in light of (i) the relatively short periods of gestation and sexual maturation in pigs; (ii) the size and frequency of litters, (iii) the
20 relatively large size of the pig which provides proportionately large yields of hemoglobin; and (iv) functional similarities between pig and human hemoglobins in the regulation of oxygen binding affinity which enables the transgenic pigs to remain
25 healthy in the presence of high levels of human hemoglobin.

The present invention also provides for recombinant nucleic acid constructs that may be used to generate transgenic pigs. In preferred
30 embodiments, such constructs place the human alpha and beta globin genes under the same promoter so as to avoid deleterious effects of globin chain imbalance and/or titration of transcription factors due to constitutive β -globin promoter activity in an
35 inappropriate cell type (e.g. a primitive erythrocyte).

In an additional embodiment, the present invention provides for a hybrid hemoglobin that comprises human α globin and pig β globin. The whole blood from transgenic pigs expressing this hybrid hemoglobin appears to exhibit a P_{50} that is advantageously higher than that of native human or pig blood.

The present invention also provides for a method of producing human hemoglobin comprising (i) introducing a human alpha globin and a human beta globin gene, under the control of a suitable promoter or promoters, into the genetic material of a pig so as to create a transgenic pig that expresses human hemoglobin in at least some of its red blood cells; (ii) collecting red blood cells from the transgenic pig; (iii) releasing the contents of the collected red blood cells; and (iv) subjecting the released contents of the red blood cells to a purification procedure that substantially separates human hemoglobin from pig hemoglobin. In a preferred embodiment of the invention, human hemoglobin may be separated from pig hemoglobin by DEAE anion exchange column chromatography.

25

4. DESCRIPTION OF THE FIGURES

Figure 1. Recombinant nucleic acid constructs.

A. Construct $\alpha\alpha\beta$ (the "116 construct"); B. Construct $\alpha\beta\beta$ (the "185" construct); C. Construct $\beta\beta\alpha$ (the "290" construct); D. Construct $\epsilon\beta\beta\alpha$; E. Construct $\zeta\beta\epsilon\alpha\beta$; F. Construct $\alpha\beta$ carrying a $\beta 108$ Asn \rightarrow Asp mutation (the "hemoglobin Yoshizuka construct"); G. Construct $\alpha\beta$ carrying a $\beta 108$ Asn \rightarrow Lys mutation (the "hemoglobin Presbyterian construct"); H. Construct $\alpha\beta(\Delta\alpha)$ coinjected with LCR α (the "285" construct); I.

35

- 9 -

Construct $\alpha\beta$ carrying an $\alpha 134$ Thr \rightarrow Cys mutation (the "227" construct); J. Construct $\alpha\beta$ carrying an $\alpha 104$ Cys \rightarrow Ser mutation (the "227" construct), a $\beta 93$ Cys \rightarrow Ala mutation, and a $\beta 112$ Cys \rightarrow Val mutation (the "228" construct); K. Construct $\alpha\delta$ (the "263" construct); and L. Construct $\alpha\delta(\Delta\alpha)$ coinjected with LCR α (the "274" construct); M. Construct LCR α coinjected with LCR $\epsilon\beta$ (the "240" construct); N. Construct $\alpha\beta$ carrying a $\beta 61$ Lys \rightarrow Met mutation (the "Hemoglobin Bologna" construct); O. Construct LCR $\epsilon\alpha\beta$ (the "318" construct); P. Construct LCR $\alpha\epsilon\beta$ (the "319" construct); Q. Construct LCR $\alpha\alpha\epsilon\beta$ (the "329" construct); R. Construct LCR $\alpha\epsilon(\beta\beta)\beta$ (the "339" construct); S. Construct $\alpha\beta$ carrying an $\alpha 75$ Asp \rightarrow Cys mutation (the "340" construct); T. Construct $\alpha\beta$ carrying an $\alpha 42$ Tyr \rightarrow Arg mutation (the "341" construct); U. Construct LCR $\epsilon\beta\alpha\alpha$ (the "343" construct); V. Construct LCR $\epsilon\beta\alpha$ (the "347" construct); W. Construct $\alpha\beta$ carrying an $\alpha 42$ Tyr \rightarrow Lys mutation; X. Construct $\alpha\beta$ carrying an $\alpha 42$ Tyr \rightarrow Arg mutation; and a $\beta 99$ Asp \rightarrow Glu mutation; Y. Construct $\alpha\beta$ carrying an $\alpha 42$ Tyr \rightarrow Lys mutation; and a $\beta 99$ Asp \rightarrow Glu mutation.

Figure 2. Transgenic pig.

Figure 3. Demonstration of human hemoglobin

expression in transgenic pigs. A. Isoelectric focusing gel analysis. B. Triton-acid urea gel of hemolysates of red blood cells representing human blood (lane 1); blood from transgenic pig 12-1 (lane 2), 9-3 (lane 3), and 6-3 (lane 4); and pig blood (lane 5) shows under-expression of human β globin relative to human α globin in the transgenic animals.

- Figure 4. Separation of human hemoglobin and pig hemoglobin by DEAE chromatography. A. Hemolyzed mixture of human and pig red blood cells; B. Hemolysate of red blood cells collected from transgenic pig 6-3. C. Human and mouse hemoglobin do not separate by DEAE chromatography under these conditions. D. Isoelectric focusing of human hemoglobin purified from pig hemoglobin.
- Figure 5. Isoelectric focussing gel of reassociated pig hemoglobin (lane 1); reassociated pig/human hemoglobin mixture (lanes 2 and 4); reassociated human hemoglobin (lane 3); and transgenic pig hemoglobin (lane 5).
- Figure 6. Separation of human hemoglobin by QCIP chromatography.
- Figure 7. Oxygen affinity of transgenic hemoglobin.

5. DETAILED DESCRIPTION OF THE INVENTION

- The present invention provides for a method of producing human hemoglobin that utilizes transgenic pigs, novel globin-encoding nucleic acid constructs, and transgenic pigs that express human hemoglobin. For purposes of clarity of description, and not by way of limitation, the detailed description of the invention is divided into the following subsections:
- (i) preparation of globin gene constructs;
 - (ii) preparation of transgenic pigs;
 - (iii) preparation of human hemoglobin and its separation from pig hemoglobin;
 - and
 - (iv) preparation of human/pig hybrid hemoglobin.

5.1. PREPARATION OF GLOBIN GENE CONSTRUCTS

The present invention provides for a method
5 of producing human globin and/or hemoglobin in
transgenic pigs. Human hemoglobin is defined herein
to refer to hemoglobin formed by globin chains encoded
human globin genes (including alpha, beta, delta,
gamma, epsilon and zeta genes) or variants thereof
10 which are naturally occurring or the products of
genetic engineering. Such variants are at least about
ninety percent homologous in amino acid sequence to a
naturally occurring human hemoglobin. In preferred
embodiments, the human hemoglobin of the invention
15 comprises a human alpha globin and a human beta globin
chain. The human hemoglobin of the invention
comprises at least two different globin chains, but
may comprise more than two chains, to form, for
example, a tetrameric molecule, octameric molecule,
20 etc. In preferred embodiments of the invention, human
hemoglobin consists of two human alpha globin chains
and two human beta globin chains. As discussed infra,
the present invention also provides for hybrid
hemoglobin comprising human α globin and pig β globin.

25 According to particular embodiments of the
present invention, at least one human globin gene,
such as a human alpha and/or a human beta globin gene,
under the control of a suitable promoter or promoters,
is inserted into the genetic material of a pig so as
30 to create a transgenic pig that carries human globin
in at least some of its red blood cells. This
requires the preparation of appropriate recombinant
nucleic acid sequences. In preferred embodiments of
the invention, both human α and human β genes are
35 expressed. In an alternative embodiment, only human α
globin is expressed. In further embodiments, human

embryonic or fetal globin genes are expressed or are used as developmental expression regulators of adult genes.

5 Human alpha and beta globin genes may be obtained from publicly available clones, e.g. as described in Swanson et al., 1992, Bio/Technol. 10:557-559. Nucleic acid sequences encoding human alpha and beta globin proteins may be introduced into
10 an animal via two different species of recombinant constructs, one which encodes human alpha globin, the other encoding human beta globin; alternatively, and preferably, both alpha and beta-encoding sequences may be comprised in the same recombinant construct.

15 A suitable promoter, according to the invention, is a promoter which can direct transcription of human alpha and beta globin genes in red blood cells. Such a promoter is preferably selectively active in erythroid cells. This would
20 include, but is not limited to, a globin gene promoter, such as the human alpha, beta, delta, epsilon or zeta promoters, or a globin promoter from another species. It may, for example, be useful to utilize pig globin promoter sequences. The human
25 alpha and beta globin genes may be placed under the control of different promoters, but, since it has been inferred that vastly different levels of globin chain production may result in lethality, it may be preferable to place the human alpha and beta globin
30 genes under the control of the same promoter sequence. In order to avoid chain imbalance and/or titration of transcription factors due to constitutive β -globin promoter activity in an inappropriate cell type, it is desirable to design a construct which leads to
35 coordinate expression of human alpha and beta globin

genes at the same time in development and at quantitatively similar levels.

In one particular, non-limiting embodiment of the invention, a construct comprising the $\alpha\alpha\beta$ construct (also termed the "116" construct; Swanson et al., 1992, Bio/Technology 10:557-559; see Figure 2A) may be utilized. Although this construct, when present as a transgene at high copy number, has resulted in deleterious effects in mice, it has been used to produce healthy transgenic pigs (see Example Section 6, *infra*).

In another particular, non-limiting embodiment of the invention, a construct comprising the $\alpha\beta$ sequence (also termed the "185" construct), as depicted in Figure 1B may be used. Such a construct has the advantage of placing both alpha and beta globin-encoding sequences under the control of the same promoter (the alpha globin promoter).

The present invention, in further specific embodiments, provides for (i) the construct $\beta\alpha$, in which the human alpha and beta globin genes are driven by separate copies of the human beta globin promoter (Figure 1C); (ii) the $\epsilon\beta\alpha$ construct, which comprises human embryonic genes zeta and epsilon under the control of the epsilon promoter and both alpha and beta genes under the control of the beta promoter (Figure 1D); (iii) the $\zeta\epsilon\alpha\beta$ construct, which comprises human embryonic genes zeta and epsilon under the control of the zeta promoter and both alpha and beta genes under the control of the alpha promoter (Figure 1E); (iv) the $\alpha\beta$ construct carrying a mutation that results in an aspartic acid residue (rather than an asparagine residue) at amino acid number 108 of β globin protein, to produce hemoglobin Yoshizuka (Figure 1F); (v) the $\alpha\beta$ construct carrying

- a mutation that results in a lysine residue (rather than an asparagine residue) at amino acid number 108 of β -globin protein, to produce hemoglobin
- 5 Presbyterian (Figure 1G); (vi) the $\alpha\beta(\Delta\alpha)$ construct, coinjected with LCR α which comprises the human β -globin gene under the control of the human α -globin promoter and a separate nucleic acid fragment comprising the human α -globin gene under its own
- 10 promoter (Figure 1H); (vii) the $\alpha\beta$ construct carrying a mutation that results in a cysteine residue (rather than a threonine residue) at amino acid number 134 of α -globin protein (Figure 1I); (viii) the $\alpha\beta$ construct carrying a mutation that results in a serine residue
- 15 (rather than a cysteine residue) at amino acid number 104 of the α -globin protein, an alanine residue (rather than a cysteine residue) at amino acid number 93 of the β -globin protein and a valine residue (rather than a cysteine residue) at amino acid number
- 20 112 of the β -globin protein (Figure 1J); (ix) the $\alpha\delta$ construct, which comprises the human adult α -globin promoter under its own promoter and the human δ -globin gene under the control of the human adult α -globin promoter (Fig. 1K); (x) Construct $\alpha\delta(\Delta\alpha)$ coinjected
- 25 with LCR α , which comprises the human δ -globin gene under the control of the human α -globin promoter and a separate nucleic acid fragment comprising the human α -globin gene under its own promoter (Fig. 1L); (xi) Construct LCR α coinjected with LCR $\epsilon\beta$, which
- 30 comprises the human α -globin gene under the control of its own promoter and a separate nucleic acid fragment comprising the human embryonic ϵ -globin gene and the adult β -globin gene under the control of their own promoters (Fig. 1M); (xii) the $\alpha\beta$ construct carrying
- 35 a mutation that results in a methionine residue (rather than a lysine residue) at amino acid number 61

of the α -globin protein (Fig. 1N); (xiii) the $\epsilon\alpha\beta$ construct, which comprises the human embryonic epsilon gene, the human adult alpha globin gene and the human adult beta globin gene linked in tandem from 5'- to 3' (Fig. 10); (xiv) the $\alpha\epsilon\beta$ construct, which comprises the human adult alpha-globin gene, the human embryonic epsilon globin gene and the human adult beta globin gene linked in tandem from 5'- to 3' (Fig. 1P); (xv) the $\alpha\alpha\epsilon\beta$ construct, which comprises two copies of the human adult alpha-globin gene, the human embryonic epsilon globin gene and the human adult beta globin gene linked in tandem from 5'- to 3' (Fig. 1Q); (xvi) the $\alpha\epsilon(\text{P}^{\text{H}}\beta\text{p})\beta$ construct, which comprises the human adult alpha-globin gene, the human embryonic epsilon globin gene and the human adult beta globin gene under the control of the endogenous porcine adult beta globin promoter all linked in tandem from 5'- to 3' (Fig. 1R); (xvii) the $\alpha\text{p}\beta$ construct carrying a mutation that results in a cysteine residue (rather than an aspartic acid residue) at amino acid number 75 of the α -globin protein (Fig. 1S); (xviii) the $\alpha\text{p}\beta$ construct carrying a mutation that results in an arginine residue (rather than a tyrosine residue) at amino acid number 42 at the α -globin protein (Fig. 1T); (xvix) the LCR $\epsilon\beta\alpha\alpha$ construct, which comprises the human embryonic epsilon globin gene, the human adult beta globin gene and two copies of the human adult alpha-globin gene linked in tandem from 5'- to 3' (Fig. 1U); (xx) the LCR $\epsilon\beta\alpha$ construct, which comprises the human embryonic epsilon globin gene, the human adult beta globin gene and the human adult alpha-globin gene linked in tandem from 5'- to 3' (Fig. 1V); (xxi) the $\alpha\text{p}\beta$ construct carrying a mutation that results in a lysine residue (rather than a tyrosine residue) at amino acid number 42 of the α -

globin protein (Fig. 1W); (xxii) the $\alpha\beta$ construct carrying a mutation that results in an arginine residue (rather than a tyrosine residue) at amino acid number 42 at the α -globin protein and a glutamic acid residue (rather than an aspartic acid residue) at amino acid number 99 of the β -globin protein (Fig. 1X); and (xxiii) the $\alpha\beta$ construct carrying a mutation that results in a lysine residue (rather than a tyrosine residue) at amino acid number 42 of the α -globin protein and a glutamic acid residue (rather than an aspartic acid residue) at amino acid number 99 of the β -globin protein (Fig. 1Y).

The recombinant nucleic acid constructs described above may be inserted into any suitable plasmid, bacteriophage, or viral vector for amplification, and may thereby be propagated using methods known in the art, such as those described in Maniatis et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y. In the working examples presented below, the PUC vector (Yanish-Perron et al., 1985, Gene 103-119) was utilized.

Constructs may desirably be linearized for preparation of transgenic pigs. Vector sequence may desirably be removed.

5.2. PREPARATION OF TRANSGENIC PIGS

The recombinant constructs described above may be used to produce a transgenic pig by any method known in the art, including but not limited to, microinjection, embryonic stem (ES) cell manipulation, electroporation, cell gun, transfection, transduction, retroviral infection, etc. Species of constructs may be introduced individually or in groups of two or more types of construct.

According to a preferred specific embodiment of the invention, a transgenic pig may be produced by the methods as set forth in Example Section 6, infra.

5 Briefly, estrus may be synchronized in sexually mature gilts (>7 months of age) by feeding an orally active progestogen (allyl trenbolone, AT: 15 mg/gilt/day) for 12 to 14 days. On the last day of AT feeding all gilts may be given an intramuscular injection (IM) of

10 prostaglandin F_{2α} (Lutalyse: 10 mg/injection) at 0800 and 1600 hours. Twenty-four hours after the last day of AT consumption all donor gilts may be administered a single IM injection of pregnant mare serum gonadotropin (PMSG: 1500 IU). Human chorionic

15 gonadotropin (HCG: 750 IU) may be administered to all donors at 80 hours after PMSG.

Following AT withdrawal, donor and recipient gilts may be checked twice daily for signs of estrus using a mature boar. Donors which exhibited estrus

20 within 36 hours following HCG administration may be bred at 12 and 24 hours after the onset of estrus using artificial and natural (respectively) insemination.

Between 59 and 66 hours after the

25 administration of HCG one- and two-cell ova may be surgically recovered from bred donors using the following procedure. General anesthesia may be induced by administering 0.5 mg of acepromazine/kg of bodyweight and 1.3 mg ketamine/kg of bodyweight via a

30 peripheral ear vein. Following anesthetization, the reproductive tract may be exteriorized following a mid-ventral laparotomy. A drawn glass cannula (O.D. 5 mm, length 8 cm) may be inserted into the ostium of the oviduct and anchored to the infundibulum using a

35 single silk (2-0) suture. Ova may be flushed in retrograde fashion by inserting a 20 g needle into the

lumen of the oviduct 2 cm anterior to the uterotubal junction. Sterile Dulbecco's phosphate buffered saline (PBS) supplemented with 0.4% bovine serum albumin (BSA) may be infused into the oviduct and flushed toward the glass cannula. The medium may be collected into sterile 17 x 100 mm polystyrene tubes. Flushings may be transferred to 10 x 60 mm petri dishes and searched at lower power (50 x) using a Wild M3 stereomicroscope. All one- and two-cell ova may be washed twice in Brinster's Modified Ova Culture-3 medium (BMOC-3) supplemented with 1.5% BSA and transferred to 50 μ l drops of BMOC-3 medium under oil. Ova may be stored at 38°C under a 90% N₂, 5% O₂, 5% CO₂ atmosphere until microinjection is performed.

One- and two-cell ova may be placed in a Eppendorf tube (15 ova per tube) containing 1 ml HEPES Medium supplemented with 1.5% BSA and centrifuged for 6 minutes at 14000 x g in order to visualize pronuclei in one-cell and nuclei in two-cell ova. Ova may then be transferred to a 5 - 10 μ l drop of HEPES medium under oil on a depression slide. Microinjection may be performed using a Laborlux microscope with Nomarski optics and two Leitz micromanipulators. 10-1700 copies of construct DNA (linearized at a concentration of about 1ng/ μ l of Tris-EDTA buffer) may be injected into one pronuclei in one-cell ova or both nuclei in two-cell ova.

Microinjected ova may be returned to microdrops of BMOC-3 medium under oil and maintained at 38°C under a 90% N₂, 5% CO₂, 5% O₂ atmosphere prior to their transfer to suitable recipients. Ova may preferably be transferred within 10 hours of recovery.

Only recipients which exhibit estrus on the same day or 24 hours later than the donors may preferably be utilized for embryo transfer.

Recipients may be anesthetized as described earlier. Following exteriorization of one oviduct, at least 30 injected one-and/or two-cell ova and 4-6 control ova
5 may be transferred in the following manner. The tubing from a 21 g x 3/4 butterfly infusion set may be connected to a 1 cc syringe. The ova and one to two mls of BMOC-3 medium may be aspirated into the tubing. The tubing may then be fed through the ostium of the
10 oviduct until the tip reaches the lower third or isthmus of the oviduct. The ova may be subsequently expelled as the tubing is slowly withdrawn.

The exposed portion of the reproductive tract may be bathed in a sterile 10% glycerol-0.9%
15 saline solution and returned to the body cavity. The connective tissue encompassing the linea alba, the fat and the skin may be sutured as three separate layers. An uninterrupted Halstead stitch may be used to close the linea alba. The fat and skin may be closed using a
20 simple continuous and mattress stitch, respectively. A topical antibacterial agent (e.g. Furazolidone) may then be administered to the incision area.

Recipients may be penned in groups of about four and fed 1.8 kg of a standard 16% crude protein
25 corn-soybean pelleted ration. Beginning on day 18 (day 0 = onset of estrus), all recipients may be checked daily for signs of estrus using a mature boar. On day 35, pregnancy detection may be performed using ultrasound. On day 107 of gestation recipients may be
30 transferred to the farrowing suite. In order to ensure attendance at farrowing time, farrowing may be induced by the administration of prostaglandin $F_{2\alpha}$ (10 mg/injection) at 0800 and 1400 hours on day 112 of gestation. In all cases, recipients may be expected
35 to farrow within 34 hours following PGF_{2a} administration.

Twenty-four hours after birth, all piglets may be processed, i.e. ears notched, needle teeth clipped, 1 cc of iron dextran administered, etc. A
5 tail biopsy and blood may also be obtained from each pig.

Pigs produced according to this method are described in Example Section 6, infra, and are depicted in Figure 2. Such pigs are healthy, do not
10 appear to be anemic, and appear to grow at a rate comparable to that of their non-transgenic littermates. Such pigs may transmit the transgene to their offspring.

Pigs having certain characteristics may be
15 especially useful for the production of human hemoglobin; such pigs, examples of which follow, represent preferred, non-limiting, specific embodiments of the invention.

According to one preferred specific
20 embodiment of the invention, a transgenic pig contains at least twenty copies of a globin transgene.

According to a second preferred specific embodiment, the P_{50} of whole blood of a transgenic pig according to the invention is increased by at least
25 ten percent over the P_{50} of the whole blood of a comparable non-transgenic pig, taking into consideration factors such as altitude, oxygen concentrations, pregnancy, the presence of mutant hemoglobin, etc. Thus, the present invention provides
30 for a non-pregnant transgenic pig that carries and expresses a human globin transgene in which the P_{50} of whole blood of the transgenic pig is at least ten percent greater than the P_{50} of whole blood of a comparable non-pregnant non-transgenic pig at the same
35 altitude.

In other preferred specific embodiments, the present invention provides for a transgenic pig in which the amount of human globin produced relative to total hemoglobin is at least two percent, more preferably at least five percent, and most preferably at least ten percent.

Section 6, infra, describes transgenic pigs which serve as working examples of preferred, non-limiting, specific examples of the invention.

5.3. PREPARATION OF HUMAN HEMOGLOBIN AND ITS SEPARATION FROM PIG HEMOGLOBIN

The present invention provides for a method for producing human hemoglobin comprising introducing a transgene or transgenes encoding human hemoglobin, such as a human alpha globin and a human beta globin gene, under the control of a suitable promoter or promoters, into the genetic material of a pig so as to create a transgenic pig that expresses human hemoglobin in at least some of its blood cells.

The present invention also provides for a method of producing human hemoglobin comprising (i) introducing a human alpha globin and a human beta globin gene, under the control of a suitable promoter or promoters, into the genetic material of a pig so as to create a transgenic pig that expresses human hemoglobin in at least some of its red blood cells; (ii) collecting red blood cells from the transgenic pig; (iii) releasing the contents of the collected red blood cells to form a lysate; (iv) subjecting the lysate of the red blood cells to a purification procedure that substantially separates human hemoglobin from pig hemoglobin; and (v) collecting the fractions that contain purified human hemoglobin. Such fractions may be identified by isoelectric focusing in parallel with appropriate standards. In a

preferred embodiment of the invention, human hemoglobin may be separated from pig hemoglobin by DEAE anion exchange column chromatography.

5 In order to prepare human hemoglobin from the transgenic pigs described above, red blood cells are obtained from the pig using any method known in the art. The red blood cells are then lysed using any method, including hemolysis in a hypotonic solution
10 such as distilled water, or using techniques as described in 1981, Methods in Enzymology Vol. 76, and/or tangential flow filtration.

For purposes of ascertaining whether human hemoglobin is being produced by a particular
15 transgenic pig, it may be useful to perform a small-scale electrophoretic analysis of the hemolysate, such as, for example, isoelectric focusing using standard techniques.

Alternatively, or for larger scale
20 purification, human hemoglobin may be separated from pig hemoglobin using ion exchange chromatography. Surprisingly, as discussed in Section 7, supra, human hemoglobin was observed to readily separate from pig hemoglobin using ion exchange chromatography whereas
25 mouse hemoglobin and human hemoglobin were not separable by such methods. Any ion exchange resin known in the art or to be developed may be utilized, including, but not limited to, resins comprising diethylaminoethyl, Q-Sepharose, QCPI (I.B.F.) Zephyr,
30 Spherox, ectiola, carboxymethylcellulose, etc. provided that the resin results in a separation of human and pig hemoglobin comparable to that achieved using DEAE resin.

According to a specific, nonlimiting
35 embodiment of the invention, in order to separate human from pig hemoglobin (including human/pig

hemoglobin hybrids) to produce substantially pure human hemoglobin, a hemolysate of transgenic pig red blood cells, prepared as above may be applied to a DEAE anion exchange column equilibrated with 0.2 M glycine buffer at pH 7.8 and washed with 0.2 M glycine pH 7.8/5 mM NaCl, and may then be eluted with a 5-30 mM NaCl gradient, or its equivalent (see, for example, Section 9 infra). Surprisingly, despite about 85 percent homology between human and pig globin chains, human and pig hemoglobin separates readily upon such treatment, with human hemoglobin eluting earlier than pig hemoglobin. Elution may be monitored by optical density at 405 nm and/or electrophoresis of aliquots taken from serial fractions. Pig hemoglobin, as well as tetrameric hemoglobin composed of heterodimers formed between pig and human globin chains, may be separated from human hemoglobin by this method. Human hemoglobin produced in a transgenic pig and separated from pig hemoglobin by this method has an oxygen binding capability similar to that of native human hemoglobin.

According to another specific, non-limiting embodiment of the invention, human hemoglobin may be separated from pig hemoglobin (including human/pig hemoglobin hybrids) using QCPI ion exchange resin as follows:

About 10 mg of hemoglobin prepared from transgenic pig erythrocytes may be diluted in 20ml of Buffer A (Buffer A = 10mM Tris, 20mM Glycine pH 7.5). This 20ml sample may then be loaded at a flow rate of about 5ml/min onto a QCPI column (10 ml) which has been equilibrated with Buffer A. The column may then be washed with 2 volumes of Buffer A, and then with 20 column volumes of a 0-50mM NaCl gradient (10 column volumes of Buffer A + 10 column volumes of 10mM Tris,

- 24 -

20mM Glycine, 50mM NaCl Ph 7.5) or, alternatively, 6
column volumes of 10mM Tris, 20mM Glycine, 15mM NaCl,
pH 7.5, and the O.D.₂₈₀ absorbing material may be
5 collected in fractions to yield the separated
hemoglobin, human hemoglobin being identified, for
example, by isoelectric focusing using appropriate
standards. The QCPI column may be cleaned by elution
with 2 column volumes of 10mM Tris, 20mM Glycine, 1M
10 NaCl, pH 7.5.

5.4. PREPARATION OF HUMAN/PIG HYBRID HEMOGLOBIN

The present invention also provides for
essentially purified and isolated human/pig hybrid
15 hemoglobin, in particular human α /pig β hybrid
hemoglobin. Pig α /human β hybrid has not been
observed to form either in vitro in reassociation
experiments or in vitro in transgenic pigs.

The present invention provides for hybrid
20 hemoglobin and its use as a blood substitute, and for
a pharmaceutical composition comprising the
essentially purified and isolated human/pig hemoglobin
hybrid in a suitable pharmacological carrier.

Hybrid hemoglobin may be prepared from
25 transgenic pigs, as described herein, and then
purified by chromatography, immunoprecipitation, or
any other method known to the skilled artisan. The
use of isoelectric focusing to separate out hemoglobin
hybrid is shown in Figures 3 and 5.

Alternatively, hybrid hemoglobin may be
30 prepared using nucleic acid constructs that comprise
both human and pig globin sequences which may then be
expressed in any suitable microorganism, cell, or
transgenic animal. For example, a nucleic acid
35 construct that comprises the human α and pig β globin
genes under the control of a suitable promoter may be

expressed to result in hybrid hemoglobin. As a specific example, human α globin and pig β globin genes, under the control of cytomegalovirus promoter, may be transfected into a mammalian cell such as a COS cell, and hybrid hemoglobin may be harvested from such cells. Alternatively, such constructs may be expressed in yeast or bacteria.

It may be desirable to modify the hemoglobin hybrid so as to render it non-immunogenic, for example, by linkage with polyethylene glycol or by encapsulating the hemoglobin in a membrane, e.g. in a liposome.

6. EXAMPLE: GENERATION OF TRANSGENIC PIGS THAT PRODUCE HUMAN HEMOGLOBIN

6.1. MATERIALS AND METHODS

6.1.1. NUCLEIC ACID CONSTRUCTS

Constructs 116 (the $\alpha\alpha\beta$ construct), 185 (the $\alpha\beta\beta$ construct), or 263 (the $\alpha\beta\delta$ construct) were microinjected into pig ova as set forth below in order to produce transgenic pigs.

6.1.2. PRODUCTION OF TRANSGENIC PIGS

Estrus was synchronized in sexually mature gilts (>7 months of age) by feeding an orally active progestogen (allyl trenbolone, AT: 15 mg/gilt/day) for 12 to 14 days. On the last day of AT feeding all gilts received an intramuscular injection (IM) of prostaglandin F_2 (Lutalyse: 10 mg/injection) at 0800 and 1600. Twenty-four hours after the last day of AT consumption all donor gilts received a single IM injection of pregnant mare serum gonadotropin (PMSG: 1500 IU). Human chorionic gonadotropin (HCG: 750 IU) was administered to all donors at 80 hours after PMSG.

Following AT withdrawal, donor and recipient gilts were checked twice daily for signs of estrus

- 26 -

using a mature boar. Donors which exhibited estrus within 36 hours following HCG administration were bred at 12 and 24 hours after the onset of estrus using
5 artificial and natural (respectively) insemination.

Between 59 and 66 hours after the administration of HCG, one- and two-cell ova were surgically recovered from bred donors using the following procedure. General anesthesia was induced
10 by administering 0.5 mg of acepromazine/kg of bodyweight and 1.3 mg ketamine/kg of bodyweight via a peripheral ear vein. Following anesthetization, the reproductive tract was exteriorized following a mid-ventral laparotomy. A drawn glass cannula (O.D. 5 mm,
15 length 8 cm) was inserted into the ostium of the oviduct and anchored to the infundibulum using a single silk (2-0) suture. Ova were flushed in retrograde fashion by inserting a 20 g needle into the lumen of the oviduct 2 cm anterior to the uterotubal
20 junction. Sterile Dulbecco's phosphate buffered saline (PBS) supplemented with 0.4% bovine serum albumin (BSA) was infused into the oviduct and flushed toward the glass cannula. The medium was collected into sterile 17 x 100 mm polystyrene tubes. Flushings
25 were transferred to 10 x 60 mm petri dishes and searched at lower power (50 x) using a Wild M3 stereomicroscope. All one- and two-cell ova were washed twice in Brinster's Modified Ova Culture-3 medium (BMOC-3) supplemented with 1.5% BSA and
30 transferred to 50 μ l drops of BMOC-3 medium under oil. Ova were stored at 38°C under a 90% N₂, 5% O₂, 5% CO₂ atmosphere until microinjection was performed.

One- and two-cell ova were placed in an Eppendorf tube (15 ova per tube) containing 1 ml HEPES
35 Medium supplemented with 1.5% BSA and centrifuged for 6 minutes at 14000 x g in order to visualize pronuclei

in one-cell and nuclei in two-cell ova. Ova were then transferred to a 5 -10 μ l drop of HEPES medium under oil on a depression slide. Microinjection was performed using a Laborlux microscope with Nomarski optics and two Leitz micromanipulators. 10-1700 copies of construct DNA (1ng/ μ l of Tris-EDTA buffer) were injected into one pronuclei in one-cell ova or both nuclei in two-cell ova.

Microinjected ova were returned to microdrops of BMOC-3 medium under oil and maintained at 38°C under a 90% N₂, 5% CO₂, 5% O₂ atmosphere prior to their transfer to suitable recipients. Ova were transferred within 10 hours of recovery.

Only recipients which exhibited estrus on the same day or 24 hours later than the donors were utilized for embryo transfer. Recipients were anesthetized as described earlier. Following exteriorization of one oviduct, at least 30 injected one- and/or two-cell ova and 4-6 control ova were transferred in the following manner. The tubing from a 21 g x 3/4 butterfly infusion set was connected to a 1 cc syringe. The ova and one to two mls of BMOC-3 medium were aspirated into the tubing. The tubing was then fed through the ostium of the oviduct until the tip reached the lower third or isthmus of the oviduct. The ova were subsequently expelled as the tubing was slowly withdrawn.

The exposed portion of the reproductive tract was bathed in a sterile 10% glycerol-0.9% saline solution and returned to the body cavity. The connective tissue encompassing the linea alba, the fat and the skin were sutured as three separate layers. An uninterrupted Halstead stitch was used to close the linea alba. The fat and skin were closed using a simple continuous and mattress stitch, respectively.

- 28 -

A topical antibacterial agent (Furazolidone) was then administered to the incision area.

Recipients were penned in groups of four and fed 1.8 kg of a standard 16% crude protein corn-soybean pelleted ration. Beginning on day 18 (day 0 = onset of estrus), all recipients were checked daily for signs of estrus using a mature boar. On day 35, pregnancy detection was performed using ultrasound. On day 107 of gestation recipients were transferred to the farrowing suite. In order to ensure attendance at farrowing time, farrowing was induced by the administration of prostaglandin $F_{2\alpha}$ (10 mg/injection) at 0800 and 1400 hours on day 112 of gestation. In all cases, recipients farrowed within 34 hours following PGF_{2a} administration.

Twenty-four hours after birth, all piglets were processed, i.e. ears were notched, needle teeth clipped, 1 cc of iron dextran was administered, etc. A tail biopsy and blood were also obtained from each pig.

6.2. RESULTS AND DISCUSSION

Of 3566 injected ova, thirteen transgenic pigs that expressed human hemoglobin were born, two of which died shortly after birth due to normal breeding-related incidents completely unrelated to the fact that they were transgenic pigs (Table I). The remaining 11 have appeared to be healthy. A photograph of one transgenic pig is presented in Figure 2. Profiles of the pigs and of the percent "authentic" and "hybrid" human hemoglobin ("HB") produced are set forth in Table II, *infra*. Total hemoglobin was calculated as the sum of human $\alpha\beta$ plus one-half of the human α pig β hybrid. Figure 3 presents the results of isoelectric focusing and

- 29 -

triton acid urea gels of hemoglobin produced by three
of these pigs (numbers 12-1, 9-3, and 6-3) which
demonstrate the expression of human alpha and beta
5 globin in these animals.

TABLE I

Efficiency of Transgenic Pig Production
Human Hemoglobin Gene Construct(s)

10

<u>Parameter</u>	<u>Total After 22 Trials</u>
Total Ova Collected	8276
Total # Fertilized	7156
15 Total # Injected	3566
# Injected Ova Transferred	3566
# Control Ova Transferred	279
# Recipients Used	104
# Pigs Born (Male, Female)	208,332
20 # Transgenic (Male, Female)	8,5 (0.36) ^a
# Expressing	13

^a Proportion of injected ova which developed into
25 transgenic pigs (13 transgenics/3566 injected ova).

30

35

SUBSTITUTE SHEET

TABLE II

FOUNDERS

PIG	GENDER	TRANSGENE CONSTRUCT	AUTHENTIC HUMAN HB	HYBRID HB	TOTAL HUMAN HB	COPY #
6-3	F	116	6.2%	8.1%	10.3%	57
9-3	F	116	1.0%	33.1%	16.6%	1
22-2	M	185	<1%	5.0%	5.0%	55
33-7	F	185	*died shortly after birth			0.5
38-1	F	185	1.0%	8.3%	5.2%	17
38-3	M	185	4.7%	17.2%	13.2%	22
38-4	M	185	3.2%	7.0%	6.7%	5
47-3	M	263	<1%	2.9%	2.0%	4-6
47-4	F	263	<1%	18.5%	10.0%	1-2
52-3	M	263	<1%	7.6%	4.0%	
52-7	M	263	<1%	26.4%	13.0%	
53-11	M	263	<1%	15.5%	8.0%	

- 31 -

Table III presents the profiles of offspring of pig number 9-3, which shows that the F1 generation of transgenic pigs are capable of expressing
5 hemoglobin. Of note, none of the offspring of pig number 6-3 were found to be transgenic, possibly due to the absence of transgene in the animal's reproductive tissue.

10

15

20

25

30

35

SUBSTITUTE SHEET

TABLE III
F1 (OFFSPRING) OF PIG 9-3

PIG	GENDER	CONST.	AUTHENTIC HUMAN HB	HYBRID HUMAN HB	TOTAL HUM.	COPY #
9-3-1	F	116	1.0%	31.5%	16.0%	1
9-3-2*	F	116	1.0%	32.9%	17.0%	1
9-3-3	M	116	1.0%	29.7%	15.0%	1
9-3-4	M	116	1.0%	32.8%	17.0%	1
9-3-6	F	116	1.0%	29.1%	15.0%	1
9-3-8	M	116	1.0%	31.6%	16.0%	1
9-3-9	M	116	1.0%	30.2%	16.0%	1

*9-3-2 died the day after birth.

The birth weights of the transgenic pigs have been approximately equivalent to the birth weights of their non-transgenic littermates. As the transgenic pigs matured, their weights remained comparable to the weights of control animals.

7. EXAMPLE: SEPARATION OF HUMAN HEMOGLOBIN FROM PIG HEMOGLOBIN BY DEAE CHROMATOGRAPHY

10 7.1. MATERIALS AND METHODS

7.1.1. PURIFICATION BY DEAE CHROMATOGRAPHY

For purification, red blood cells were collected by centrifugation of 5000 rpm for 3 minutes in an eppendorf microcentrifuge and washed three times with an equal volume (original blood) of 0.9% NaCl. Red cells were lysed with 1.5 volumes deionized H₂O, centrifuged at 15,000 rpm, and the supernatant was fractionated by anion exchange chromatography. DEAE cellulose chromatography (DE-SE manufactured by Whatman, Ltd.) was performed according to W. A. Schroeder and T. H. J. Huisman "The Chromatography of Hemoglobin", Dekker, New York, pp. 74-77. The 0.25 ml red cell hemolysate described above was applied to 1 cm x 7 cm DE-52 column pre-equilibrated in 0.2 M glycine pH 7.8 and was washed with 5 column volumes of 0.2 M glycine pH 7.8/5 mM NaCl. Hemoglobins were eluted with a 200 ml 5-30 mM NaCl/0.2 M glycine pH 7.8 gradient. To complete elution of pig hemoglobin, an additional 50 to 100 ml of 30 mM CaCl₂/glycine pH 7.8 was added to the column. Elution of hemoglobin was monitored by absorbance of 415 mμ and by IEF analysis of column fractions.

7.1.2. REASSOCIATION OF GLOBIN CHAINS

Reassociation of globin chains was performed essentially as described in Methods in Enzymol.

- 34 -

76:126-133. 25 lambda of pig blood, 25 lambda of human blood, or a 25 lambda mixture of 12.5 lambda human blood and 12.5 lambda pig blood were treated as follows. The blood was pelleted at a setting of 5 on microfuge for 2 minutes, then washed three times with 100 lambda 0.9 percent NaCl. The cells were lysed with 50 lambda H₂O, then spun at high speed to confirm lysis. 50 lambda of the lysed cells was then combined with 50 lambda 0.2 M Na Acetate, pH 4.5, put on ice and then incubated in a cold room overnight. After adding 1.9 ml 0.1 M NaH₂PO₄, pH 7.4 each sample was spun in centricon tubes at 4°C and 5K until about 0.5 ml remained. Then 1 ml of 0.1 M NaH₂PO₄ pH 7.4 was added and spun through at about 5K until about 0.2 ml volume was left. The hemoglobin was then washed from the walls of the centricon tube, an eppendorf adaptor was attached, and a table top microfuge was used to remove each sample from its centricon tube. The samples were then analyzed by isoelectric focusing.

7.2. RESULTS AND DISCUSSION

7.2.1. HUMAN AND PIG HEMOGLOBIN WERE SEPARATED FROM A HEMOLYZED MIXTURE OF HUMAN AND PIG BLOOD

Equal proportions of human and of pig blood were mixed and lysed, and the resulting hemolysate was subjected to DEAE chromatography as described supra. As shown in Figure 4A, pig hemoglobin separated virtually completely from human hemoglobin. This complete separation is surprising in light of the structural similarity between human and pig hemoglobin; pig and human alpha globin chains are 84.4 percent homologous and pig and human beta globin chains are 84.9 percent homologous. It is further surprising because, as shown in Figure 4C, when human and mouse blood was mixed, hemolyzed, applied to and

eluted from a DEAE column according to methods set forth in Section 7.1.1., supra, human and mouse hemoglobin were not observed to separate despite the fact that mouse and human alpha globin chains are about 85.8 percent homologous and mouse and human beta globin chains are 80.1 percent homologous. The ease of separation of human and pig hemoglobin on DEAE resin appears to be both efficient and economical.

Interestingly, the order of elution of the proteins from the anion exchange column was not as expected. Based on the relative pI's of the proteins as deduced from the IEF gels, the predicted order of elution would be first the hybrid (human α /pig β) followed by the authentic human α /human β . The last protein to elute from the anion exchange column then would be the endogenous pig α /pig β protein. However, under all the conditions currently attempted the order of elution was altered such that the human hemoglobin was the first to elute. The second peak was an enriched fraction of the hybrid followed very closely by the pig hemoglobin.

7.2.2. HUMAN AND PIG HEMOGLOBIN AND HUMAN/PIG HETEROLOGOUS HEMOGLOBIN WERE SEPARATED FROM HEMOLYSATE PREPARED FROM A TRANSGENIC PIG

Blood from transgenic pig 6-3 (as described in Section 6, supra) was lysed by hypotonic swelling and the resulting hemolysate was subjected to DEAE chromatography as described supra. As shown in Figure 4B, human hemoglobin was separated from pig hemoglobin and from human α globin/pig β globin heterologous hemoglobin. As shown in Figure 4D, human hemoglobin was substantially purified by this method.

7.2.3. PIG ALPHA GLOBIN/HUMAN BETA GLOBIN
HETEROLOGOUS HEMOGLOBIN DOES NOT
APPEAR TO FORM BASED ON REASSOCIATION
DATA

5 Heterologous association between pig alpha
globin and human beta-globin chains has not been
detected in hemolysates obtained from human
hemoglobin-expressing transgenic pigs. It was
10 possible, however, that this observation could be
explained by relatively low levels of human beta
globin expression. Alternatively, association between
pig alpha globin and human beta globin may be
chemically unfavorable. In order to explore this
15 possibility, reassociation experiments were performed
in which pig and human hemoglobin were mixed,
dissociated, and then the globin chains were allowed
to reassociate. As shown in the isoelectric focusing
gels depicted in Figure 5, although pig α /pig β , human
20 α /human β , and human α /pig β association was observed,
no association between pig α globin and human β globin
appeared to have occurred. Therefore the pig α /human
 β heterologous hemoglobin should not be expected to
complicate the purification of human hemoglobin from
25 transgenic pigs.

8. EXAMPLE: SEPARATION OF HUMAN
HEMOGLOBIN FROM PIG HEMOGLOBIN
BY QCPI CHROMATOGRAPHY

8.1. MATERIALS AND METHODS

30 Clarified hemolysate from transgenic pig 6-3
13mg/ml; Buffer A: 10mM Tris, 20mM Glycine pH 7.5;
Buffer B: 10mM Tris, 20mM Glycine, 15 mM NaCl pH 7.5;
Buffer C: 10mM Tris, 20mM Glycine, 1M NaCl pH 7.5;
Buffer D: 10mM Tris, 20mM Glycine, 50 mM NaCl pH 7.5;
35 QCPI column 10ml Equilibrated in Buffer A; Trio
purification system. 10mg of hemoglobin prepared from

transgenic pig 6-3 was diluted in 20ml Buffer A. 20ml of sample was loaded at a flow rate of 5ml/min onto the QCPI column, and washed with 2 column volumes of Buffer A. The column was then washed with 20 column volumes of a 0-50mM NaCl gradient. (10 column volumes Buffer A + 10 column volumes of Buffer D) and the O.D.₂₈₀ absorbing material was collected. The column was then cleaned with 2 column volumes of Buffer C, and then re-equilibrated with 2 column volumes of Buffer A.

8.2. RESULTS

Analysis of the UV trace (peak vs. volume of gradient) (Fig. 6) revealed that the human hemoglobin was eluted at 15 mM NaCl. Subsequent purifications have been performed utilizing the same protocol as above, only using 6 column volumes of Buffer B (15mM NaCl) to elute the human hemoglobin rather than the gradient. In addition, non-transgenic pig chromatographed by this method does not elute from the QCPI with Buffer B, while native human hemoglobin does. The protein that eluted at 15mM NaCl was analyzed on the Resolve isoelectric focussing system and found to be essentially pure of contaminating pig hemoglobin or hybrid hemoglobin.

9. EXAMPLE: HUMAN ALPHA/PIG BETA GLOBIN HYBRID HEMOGLOBIN EXHIBIT INCREASED P_{50}

As shown in Tables II and III, supra, transgenic pigs of the invention were all found to produce significant amounts of human α /pig β globin hybrid hemoglobin (the pig α /human β hybrid was not observed). Significantly, pigs that expressed higher percentages of hybrid also appeared to exhibit elevated P_{50} values for their whole blood (Figure 7).

Various publications are cited herein which are hereby incorporated by reference in their entirety.

5

10

15

20

25

30

35

SUBSTITUTE SHEET

WHAT IS CLAIMED IS:

1. A transgenic pig that (i) comprises a nucleic acid construct encoding human α globin and
5 (ii) expresses human α globin in at least some of its red blood cells.
2. A transgenic pig that (i) comprises a nucleic acid construct encoding human α globin and a
10 nucleic acid construct encoding human β globin and (ii) expresses human α globin and human β globin in at least some of its red blood cells.
3. A transgenic pig that (i) comprises a
15 nucleic acid construct encoding human α globin and human β globin and (ii) expresses human α globin and human β globin in at least some of its red blood cells.
- 20 4. The transgenic pig of claim 1 in which the nucleic acid construct is the LCR α construct.
5. The transgenic pig of claim 2 in which the nucleic acid constructs are the LCR α and LCR $\epsilon\beta$
25 constructs.
6. The transgenic pig of claim 3 in which the nucleic acid construct is the 116 construct as depicted in Figure 1A.
30
7. The transgenic pig of claim 3 in which the nucleic acid construct is the 185 construct as depicted in Figure 1B.
35

8. The transgenic pig of claim 3 in which the nucleic acid construct is the $\beta\alpha$ construct as depicted in Figure 1C.

5

9. The transgenic pig of claim 3 in which the nucleic acid construct is the hemoglobin Yoshizuka construct as depicted in Figure 1F.

10

10. The transgenic pig of claim 3 in which the nucleic acid construct is the hemoglobin Presbyterian construct as depicted in Figure 1G.

15

11. The transgenic pig of claim 3 in which the nucleic acid construct is the $\alpha\beta(\Delta\alpha)$ construct as depicted in Figure 1H.

20

12. The transgenic pig of claim 3 in which the nucleic acid construct is the 227 construct as depicted in Figure 1I.

25

13. The transgenic pig of claim 3 in which the nucleic acid construct is the 228 construct as depicted in Figure 1J.

30

14. The transgenic pig of claim 3 in which the Hemoglobin Bologna construct is the 228 construct as depicted in Figure 1N.

15. The transgenic pig of claim 3 in which the nucleic acid construct is the 318 construct as depicted in Figure 1O.

35

16. The transgenic pig of claim 3 in which the nucleic acid construct is the 319 construct as depicted in Figure 1P.

17. The transgenic pig of claim 3 in which
the nucleic acid construct is the 329 construct as
5 depicted in Figure 1Q.

18. The transgenic pig of claim 3 in which
the nucleic acid construct is the 339 construct as
depicted in Figure 1R.

10

19. The transgenic pig of claim 3 in which
the nucleic acid construct is the 340 construct as
depicted in Figure 1S.

15

20. The transgenic pig of claim 3 in which
the nucleic acid construct is the 341 construct as
depicted in Figure 1T.

21. The transgenic pig of claim 3 in which
20 the nucleic acid construct is the 343 construct as
depicted in Figure 1U.

22. The transgenic pig of claim 3 in which
the nucleic acid construct is the 347 construct as
25 depicted in Figure 1V.

23. The transgenic pig of claim 3 in which
the nucleic acid construct is as depicted in Figure
1W.

30

24. The transgenic pig of claim 3 in which
the nucleic acid construct is as depicted in Figure
1X.

35

25. The transgenic pig of claim 3 in which the nucleic acid construct is as depicted in Figure 1Y6.

5

26. A transgenic pig that (i) comprises a nucleic acid construct encoding human δ globin and (ii) expresses human δ globin in at least some of its red blood cells.

10

27. The transgenic pig of claim 3 in which the nucleic acid construct is the 263 construct as depicted in Figure 1K.

15

28. The transgenic pig of claim 3 in which the nucleic acid construct is the 274 construct as depicted in Figure 1L.

20

29. The transgenic pig of claim 1, 2 or 3 which comprises, in a single cell, at least twenty copies of a globin transgene.

30. The transgenic pig of claim 1, 2 or 3 in which the P_{50} of the whole blood of the transgenic pig, when non-pregnant, is at least ten percent greater than the P_{50} of whole blood of a non-pregnant non-transgenic pig at the same altitude.

25

31. The transgenic pig of claim 1, 2 or 3 in which the amount of human globin produced relative to total hemoglobin is at least two percent.

30

32. The transgenic pig of claim 1, 2 or 3 in which the amount of human globin produced relative to total hemoglobin is at least five percent.

35

33. The transgenic pig of claim 1, 2 or 3 in which the amount of human globin produced relative to total hemoglobin is at least ten percent.

5

34. A method of purifying human hemoglobin from a mixture of human hemoglobin, pig hemoglobin, and human/pig hybrid hemoglobin, comprising:

- 10 (i) collecting red blood cells from a transgenic pig according to claim 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25;
- 15 (ii) releasing the contents of the collected red blood cells to produce a lysate;
- (iii) applying the lysate of step (ii) to a DEAE anion exchange column equilibrated with 0.2M glycine at a pH of 7.8;
- 20 (iv) eluting the column with a 5-30 mM NaCl gradient; and
- (v) collecting the fractions that
- 25 contain purified human hemoglobin.

35. A method of purifying human hemoglobin from a mixture of human hemoglobin, pig hemoglobin, and human/pig hybrid hemoglobin, comprising:

- 30 (i) collecting red blood cells from a transgenic pig according to claim 2, 3, 5, 6, ~~7~~, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25;

35

- (ii) releasing the contents of the collected red blood cells to produce a lysate;
- 5 (iii) applying the lysate of step (ii) to a QCIP column equilibrated with 10mM Tris, 20mM Glycine pH 5.0;
- (iv) eluting the column with 6 column volumes of 10mM Tris, 20mM Glycine, 15mM NaCl, pH 7.5; and
- 10 (v) collecting the fractions that contain purified hemoglobin.

36. An essentially purified and isolated
15 human/pig hemoglobin hybrid comprising human α globin and pig β globin.

37. A nucleic acid construct comprising a human α globin gene and a pig beta globin gene under
20 the control of suitable promoter sequences.

38. A pharmaceutical composition comprising the essentially purified and isolated human/pig hemoglobin hybrid of claim 36 in a suitable
25 pharmacological carrier.

30

35

1/35

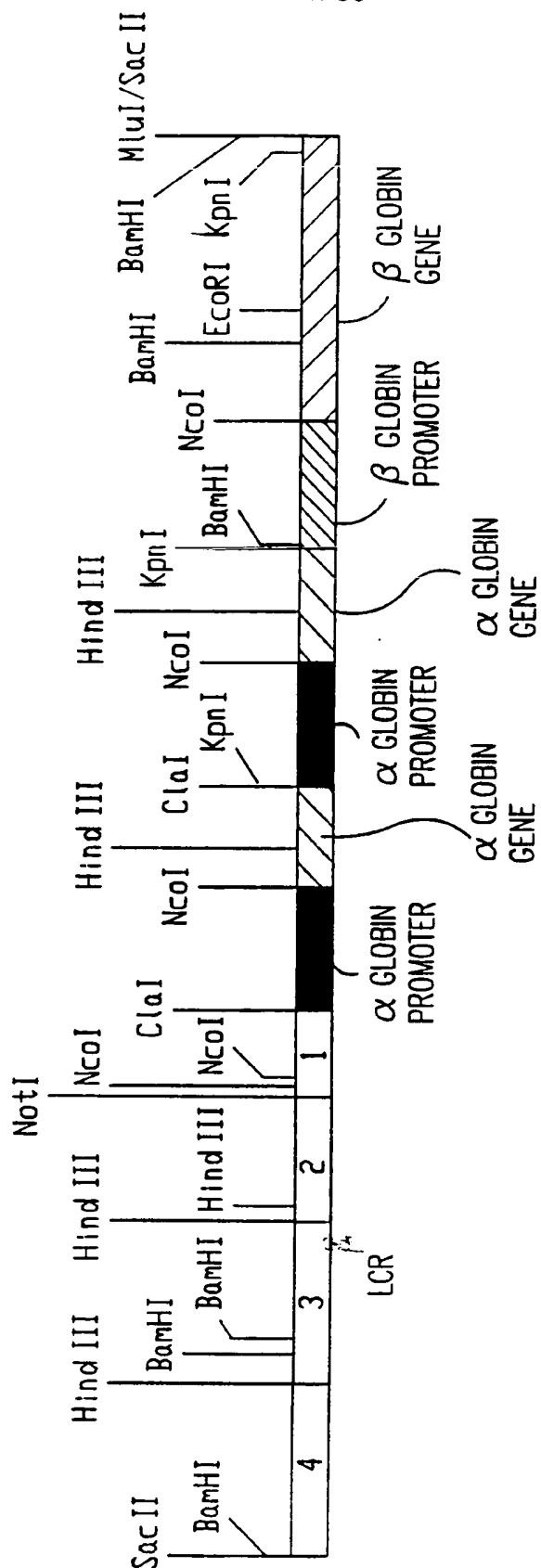


FIG.1A

2/35

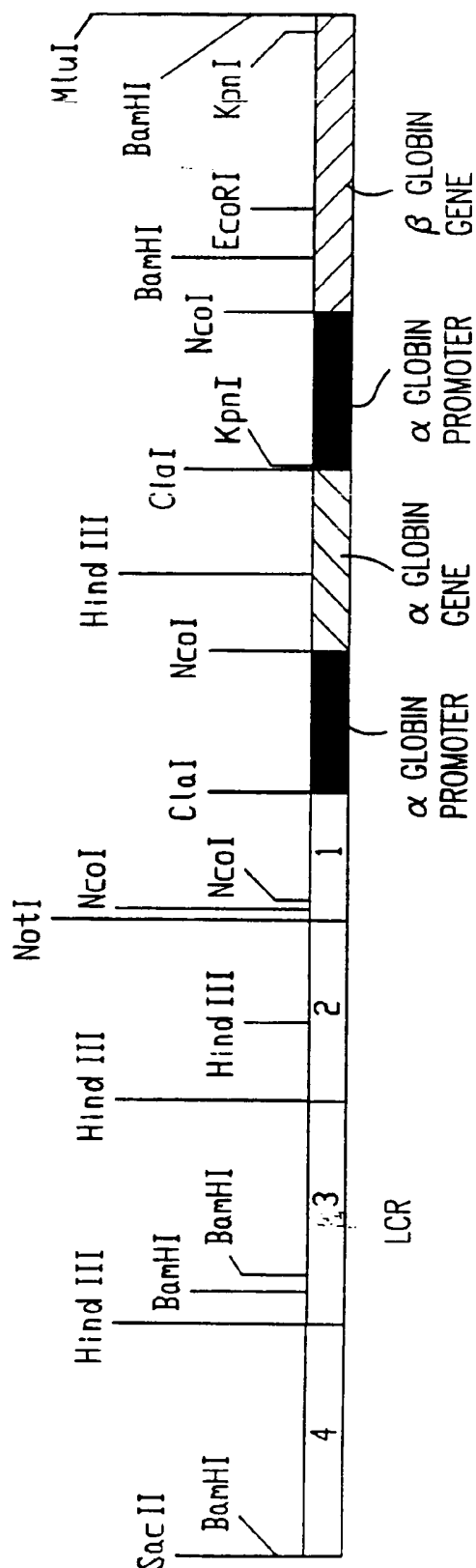


FIG.1B

3 / 35

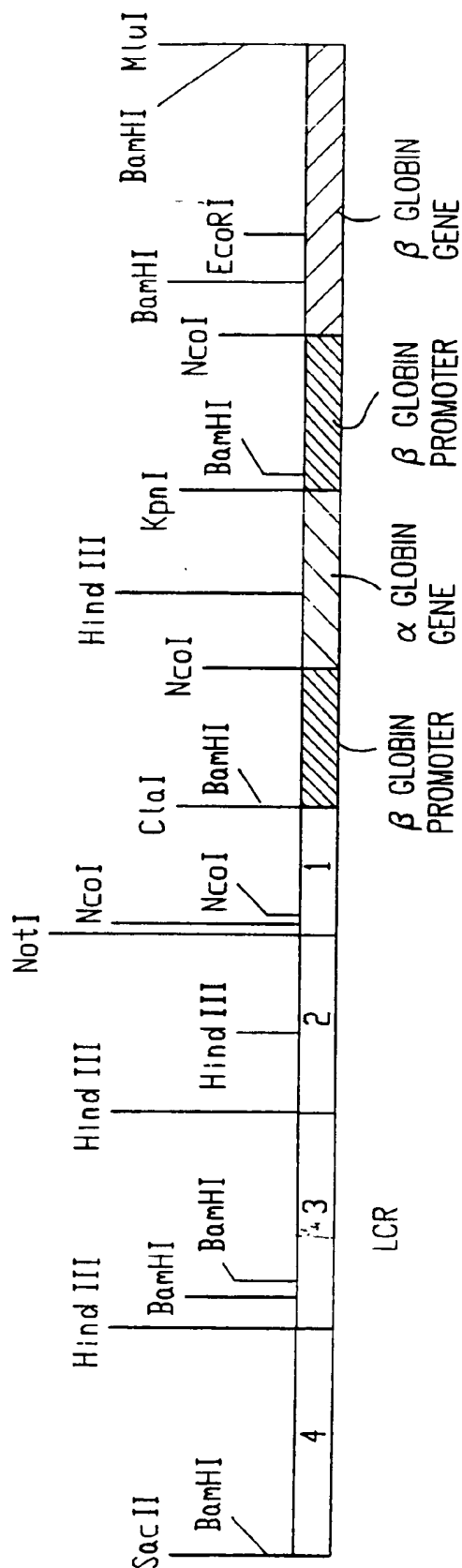


FIG.1C

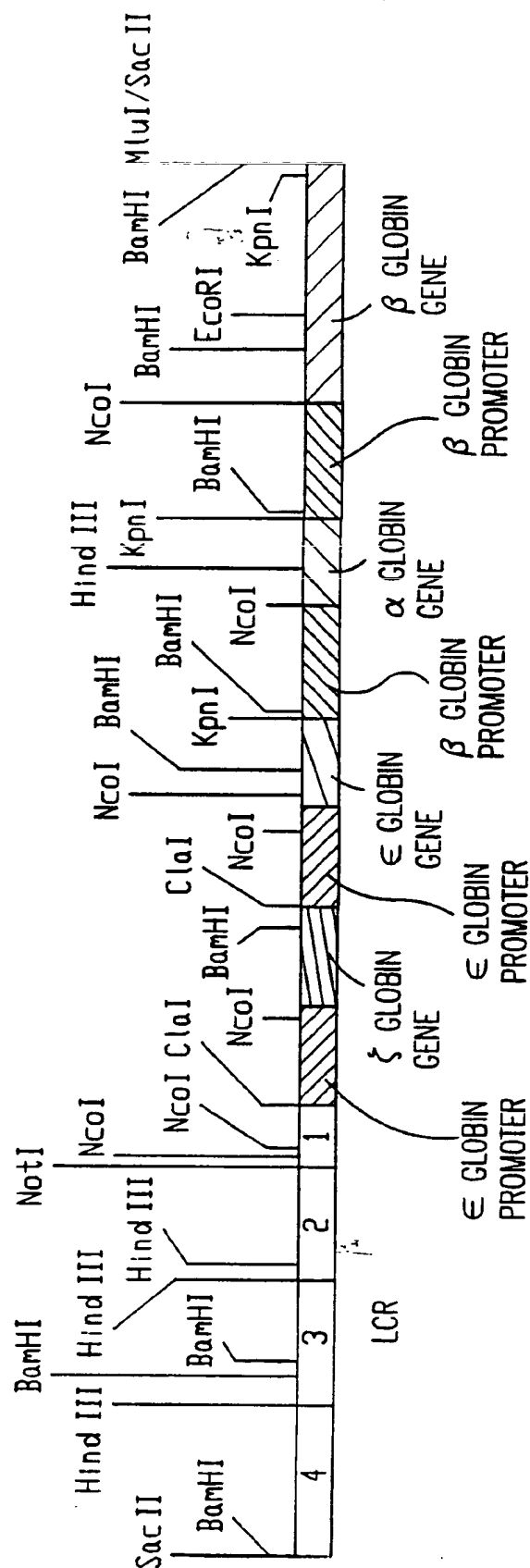


FIG. 1D

5/35

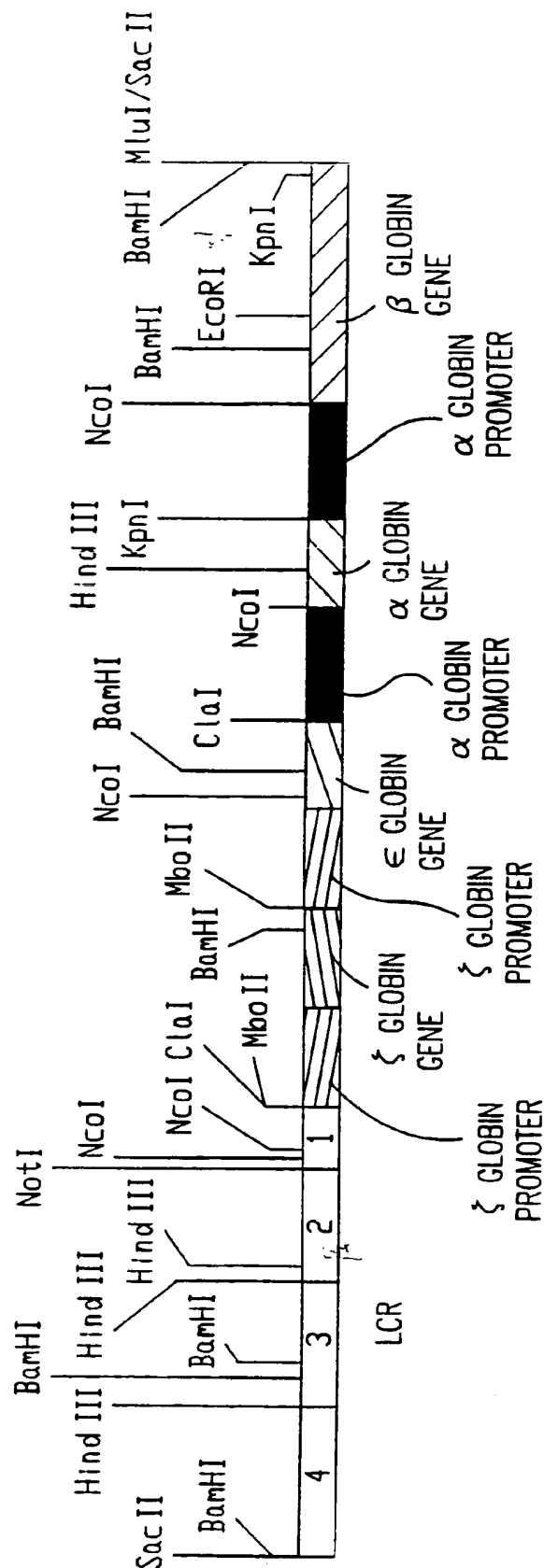


FIG. 1E

6/35

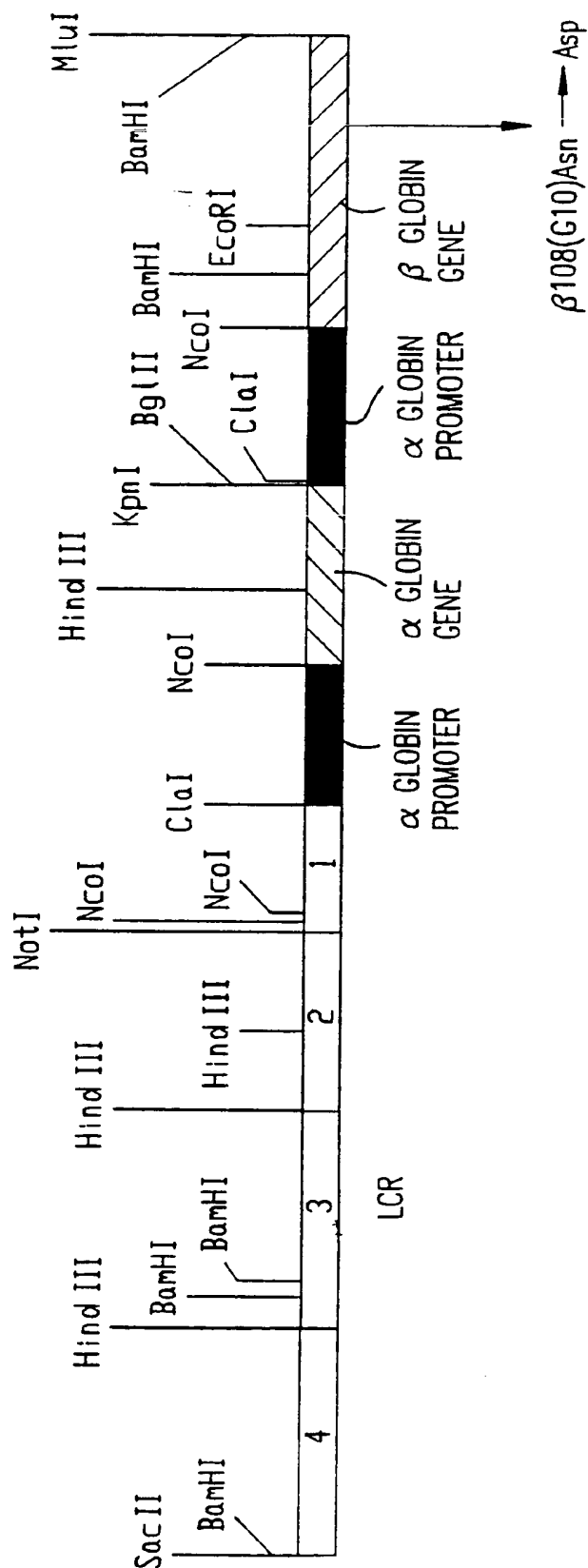


FIG.1F

7/35

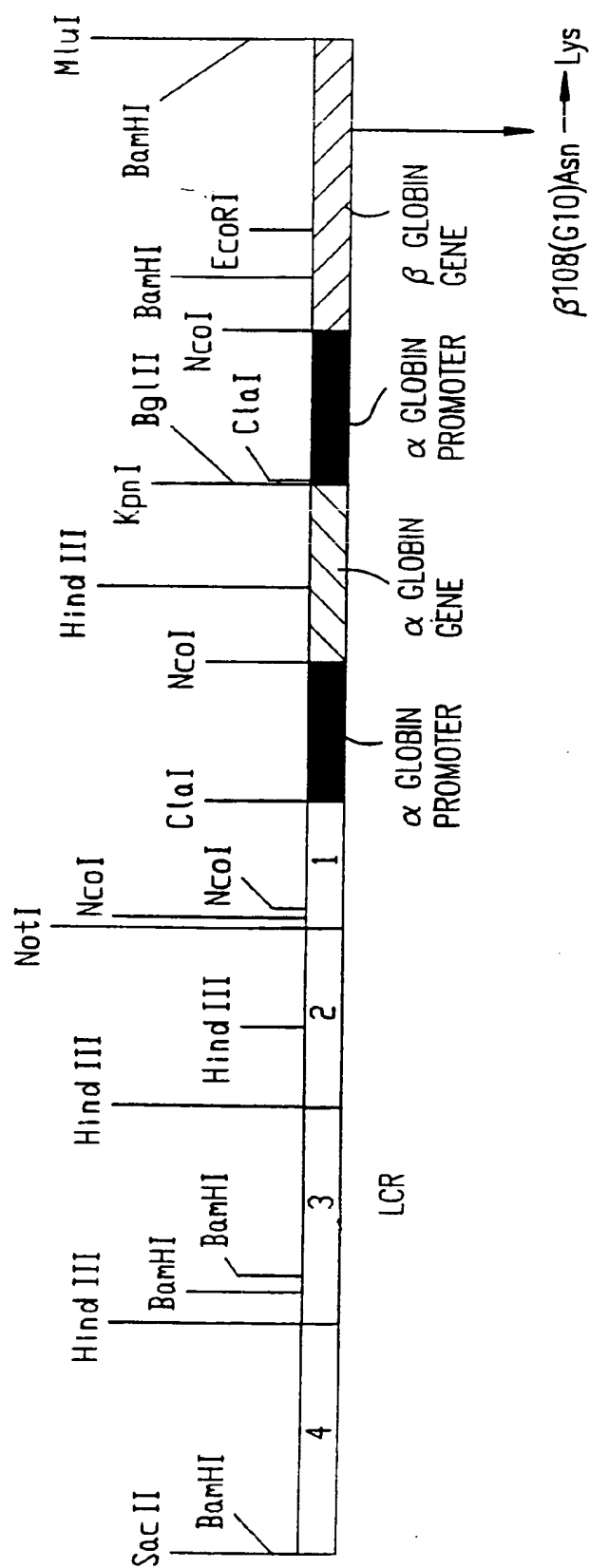


FIG.1G

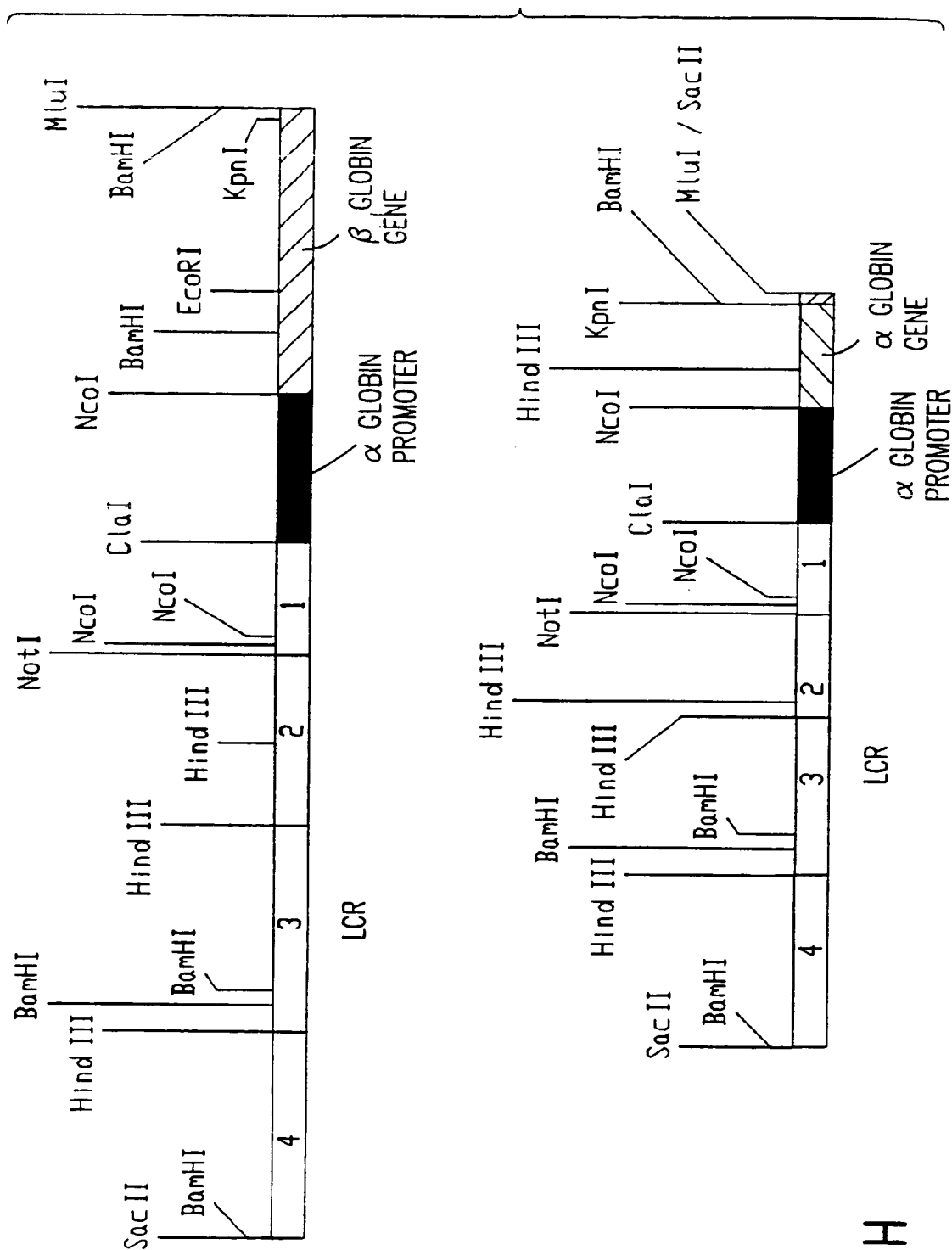


FIG.1H

9/35

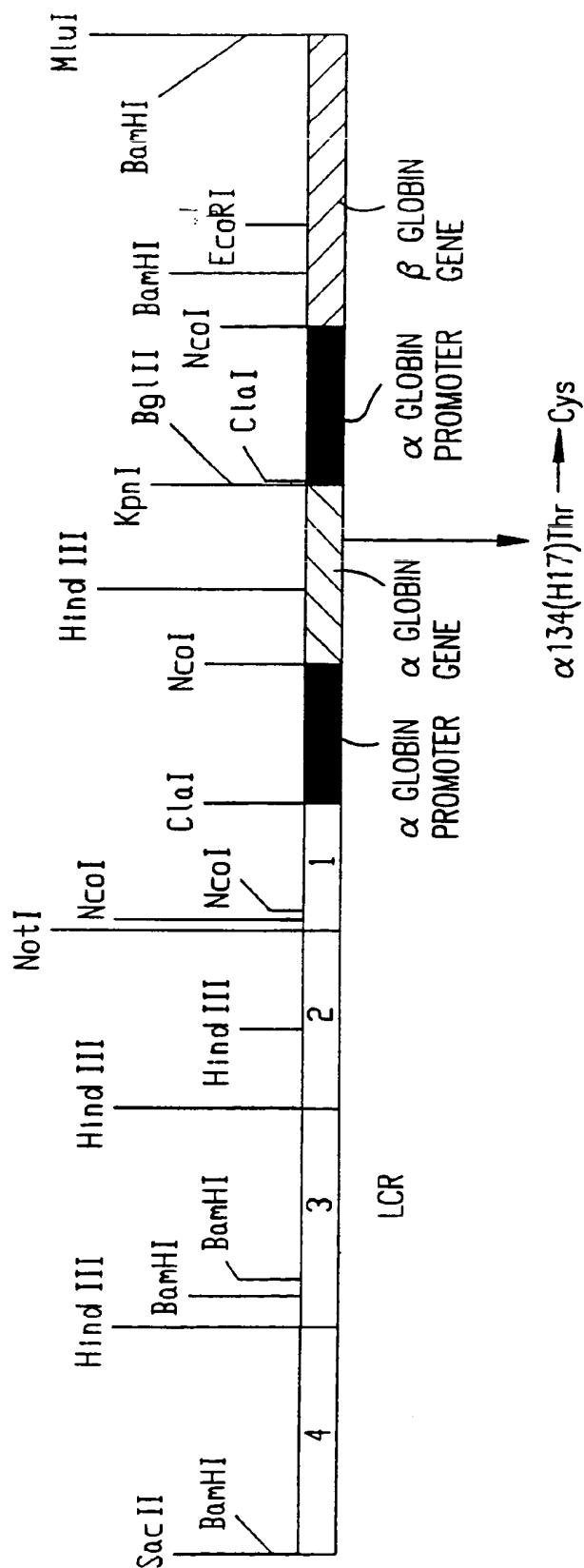


FIG.11

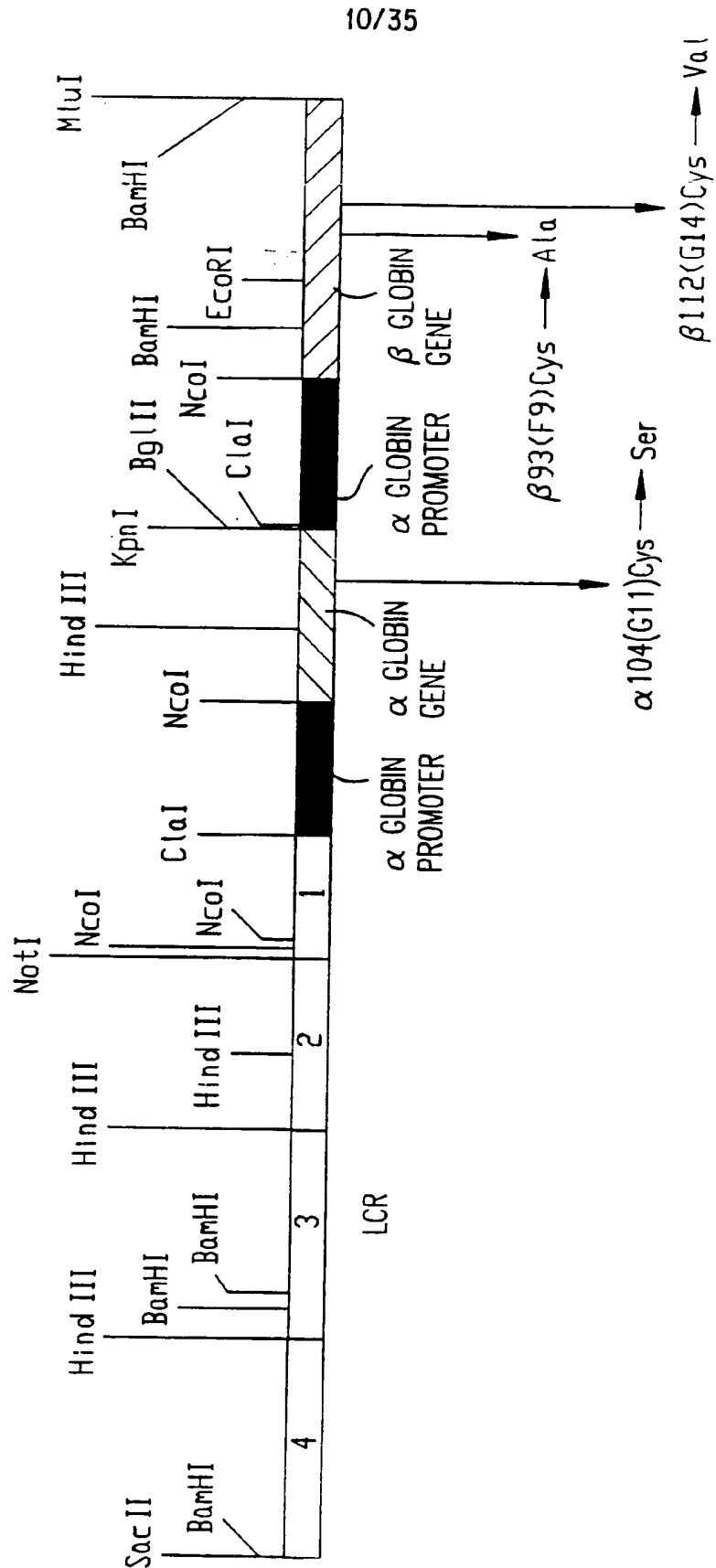


FIG.1J

11/35

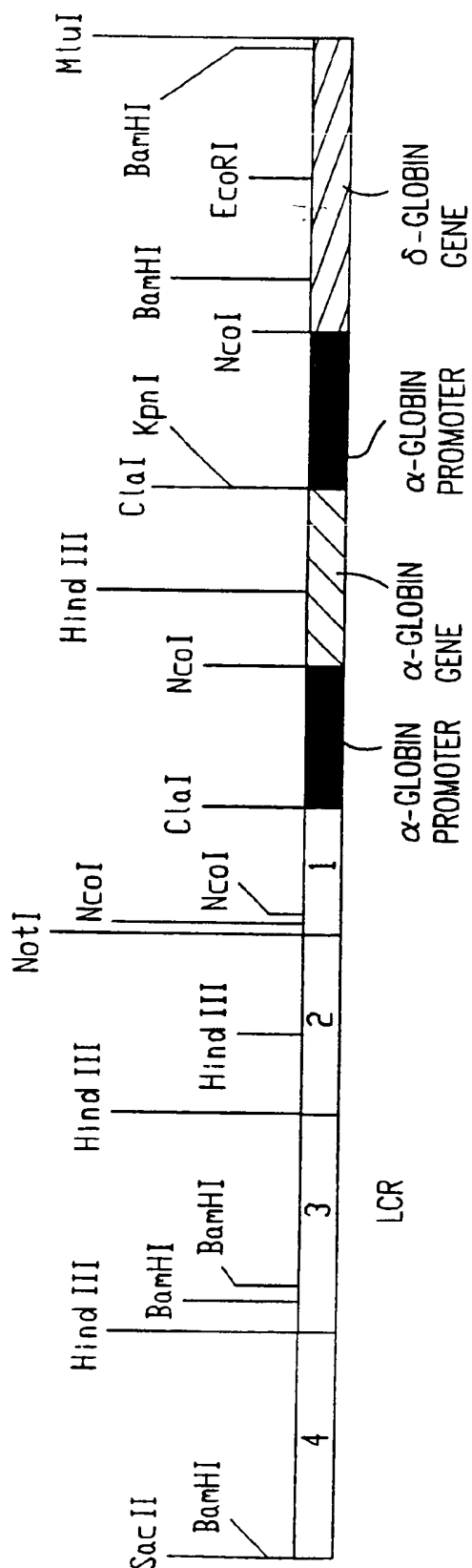


FIG.1K

12/35

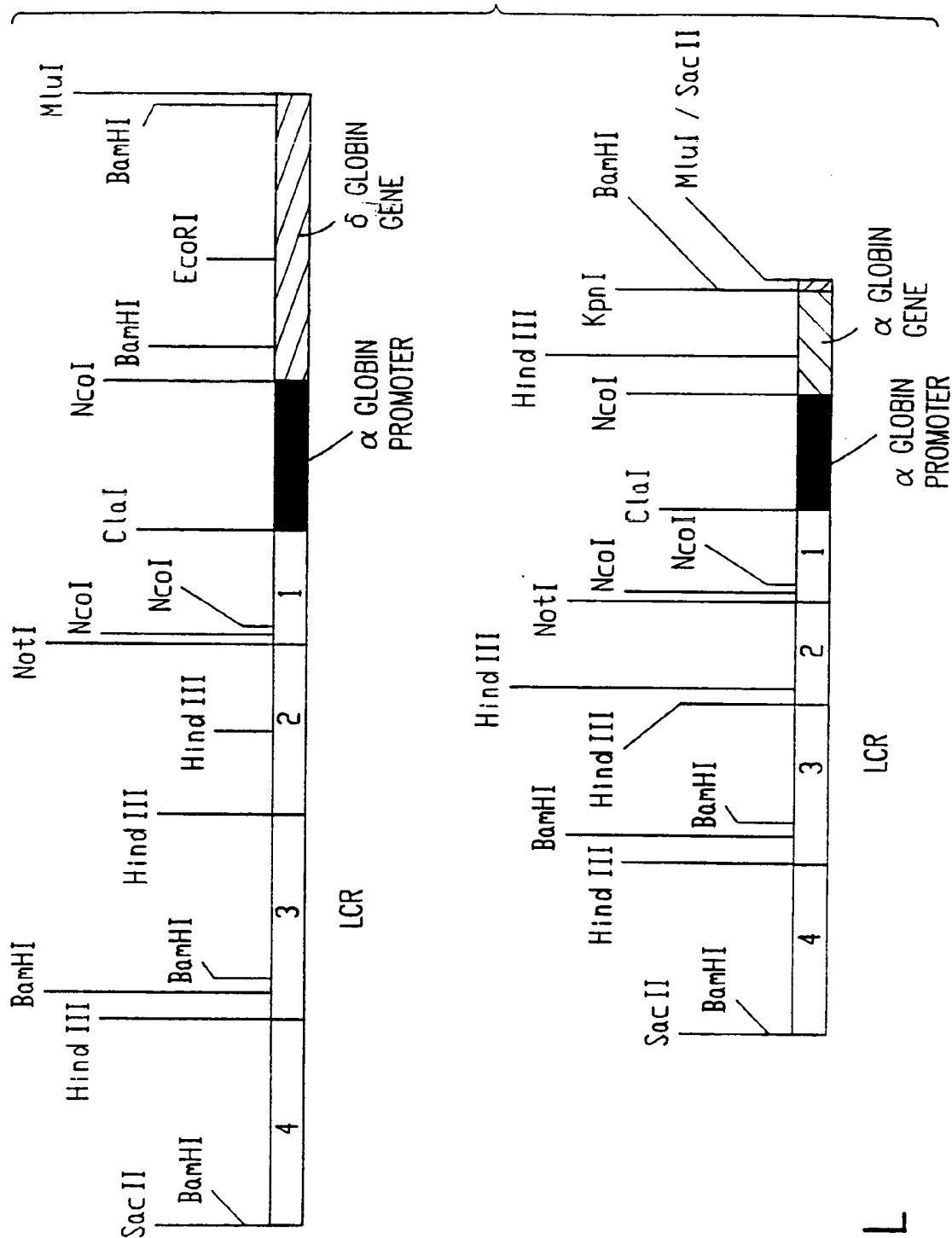


FIG.1L

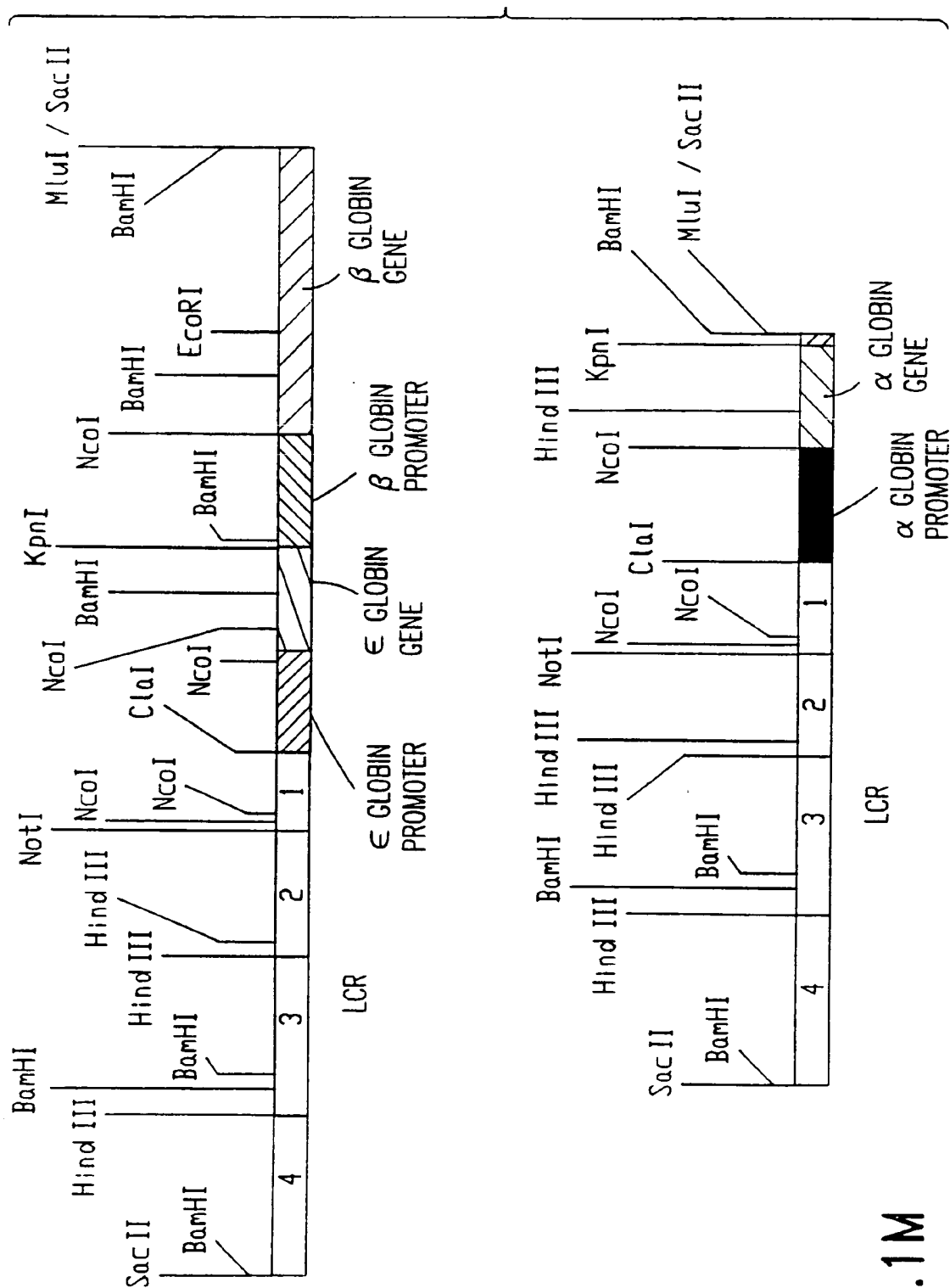


FIG. 1M

14/35

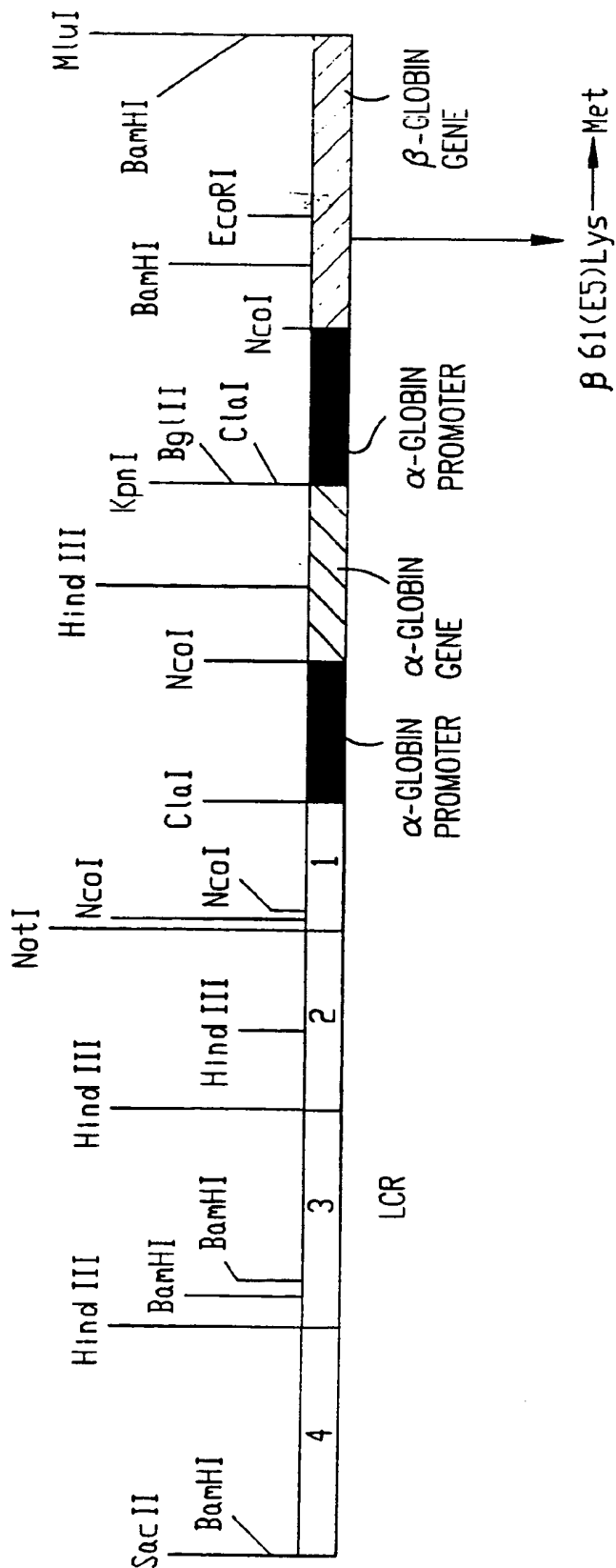


FIG.1N

15/35

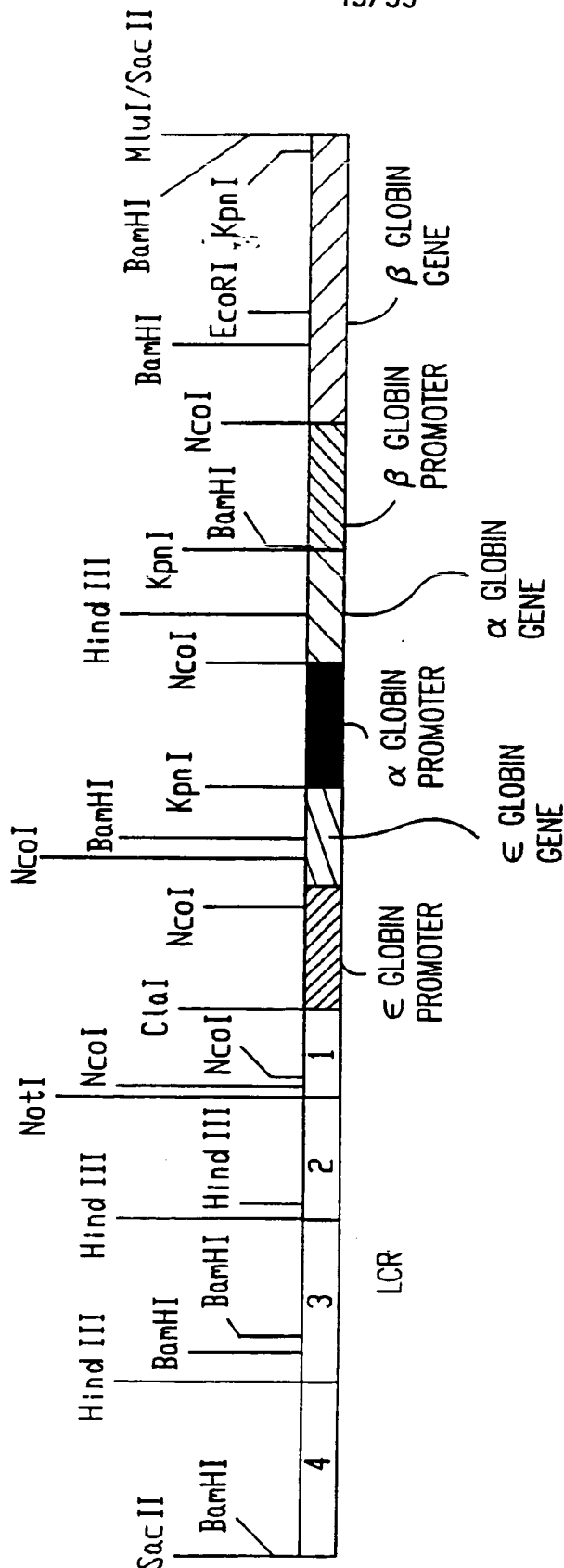


FIG.10

16/35

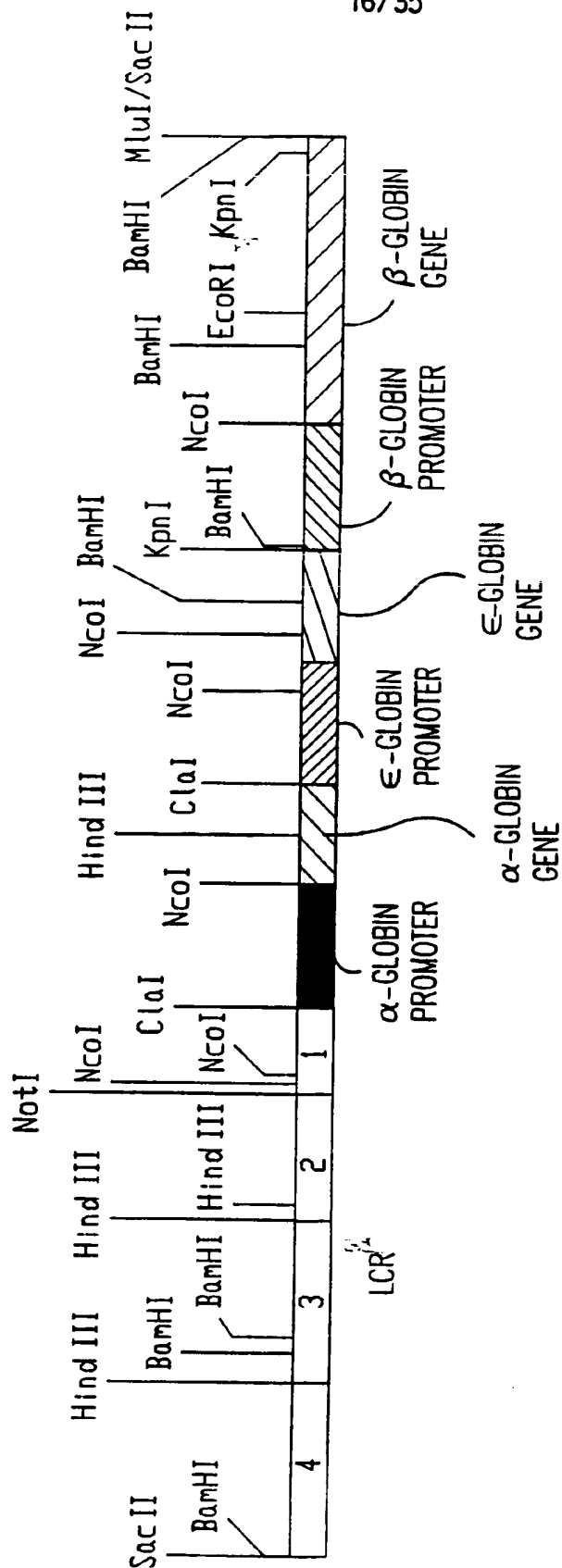


FIG.1P

17/35

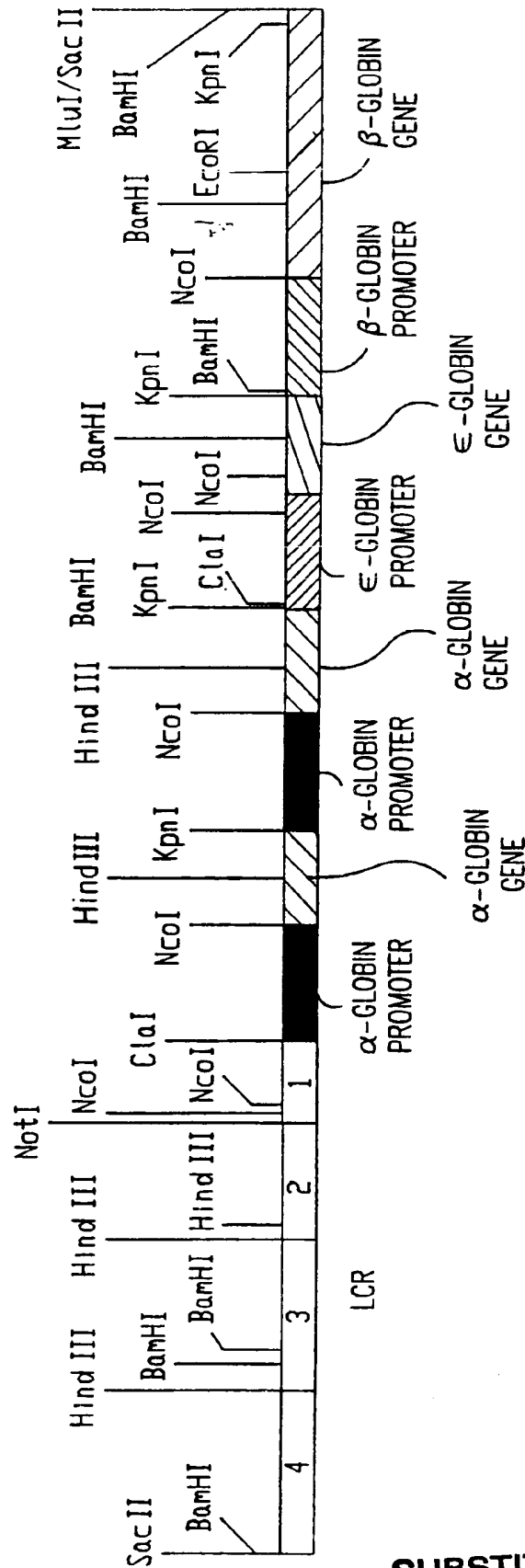


FIG. 1Q

SUBSTITUTE SHEET

18/35

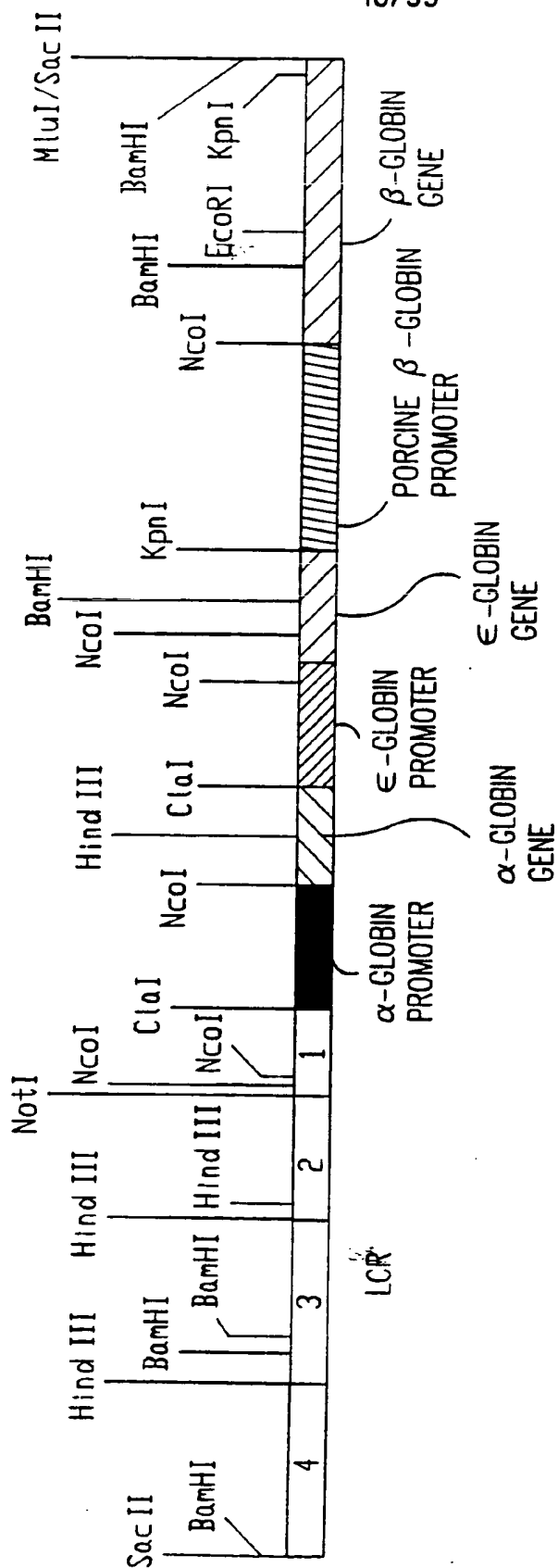


FIG.1R

19/35

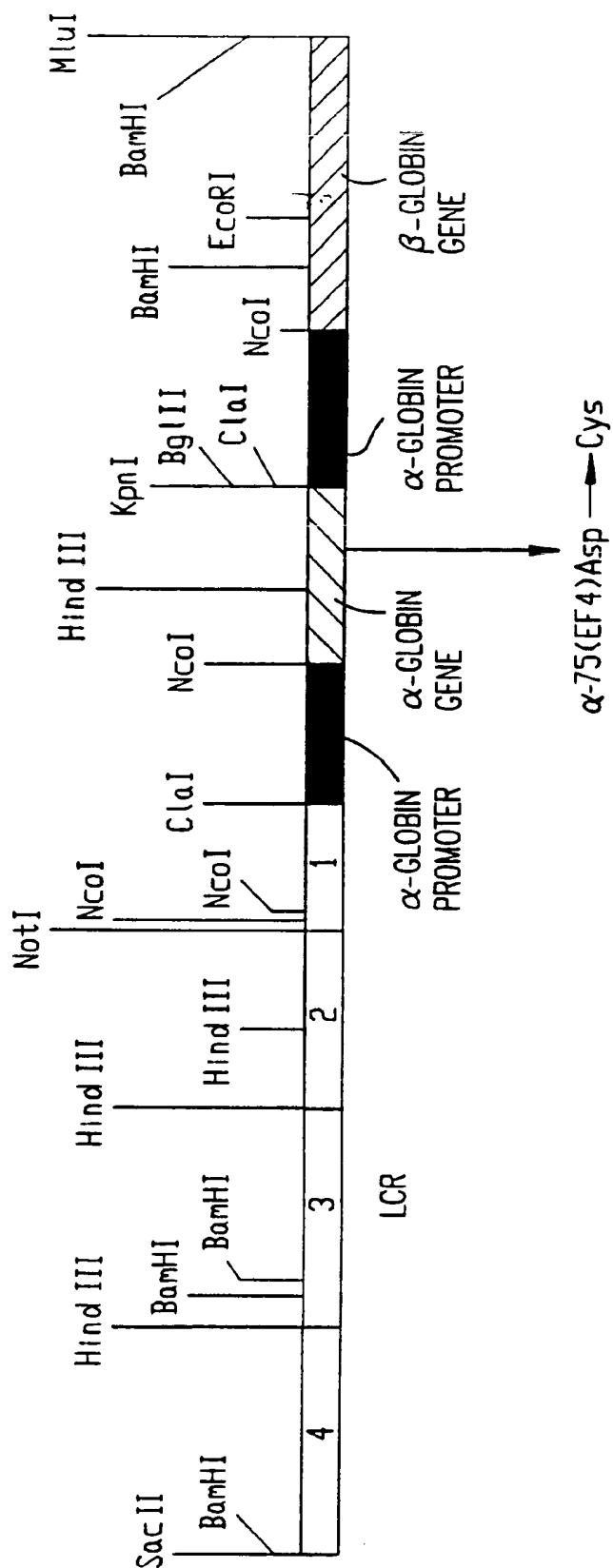


FIG.1S

20/35

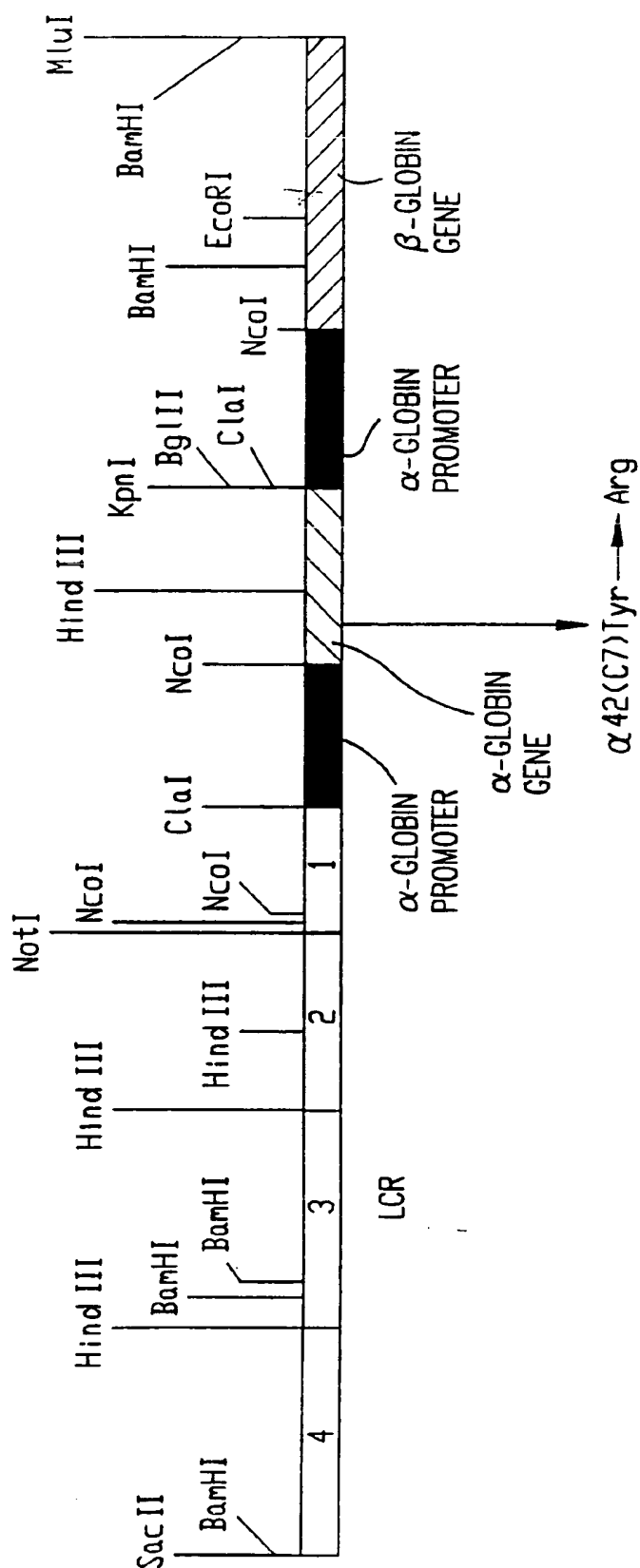


FIG.1T

SUBSTITUTE SHEET

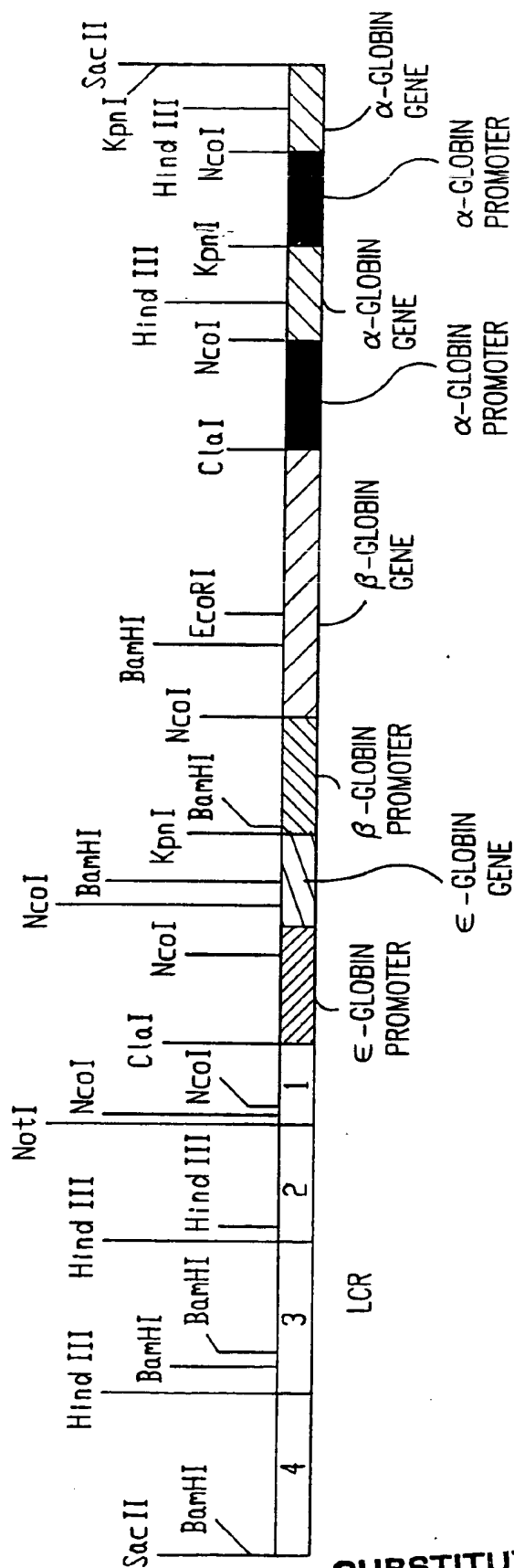


FIG. 1U

SUBSTITUTE SHEET

22 / 35

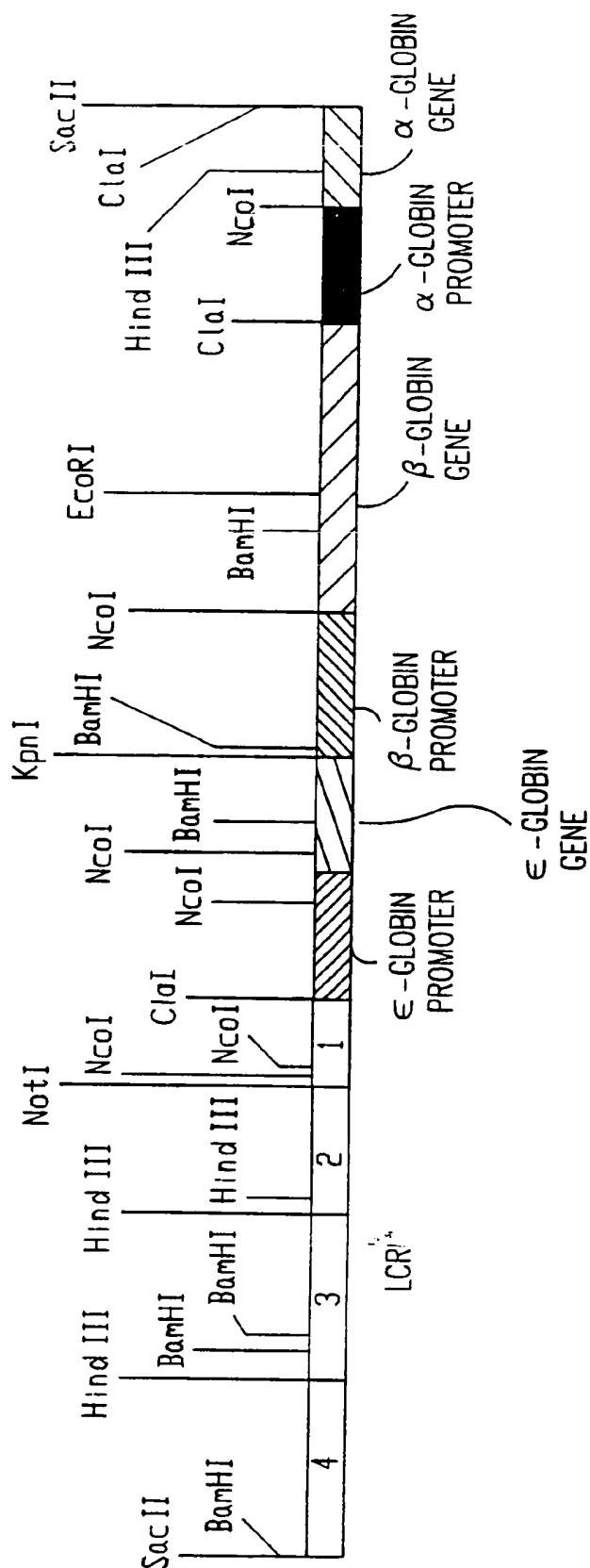


FIG.1V

23/35

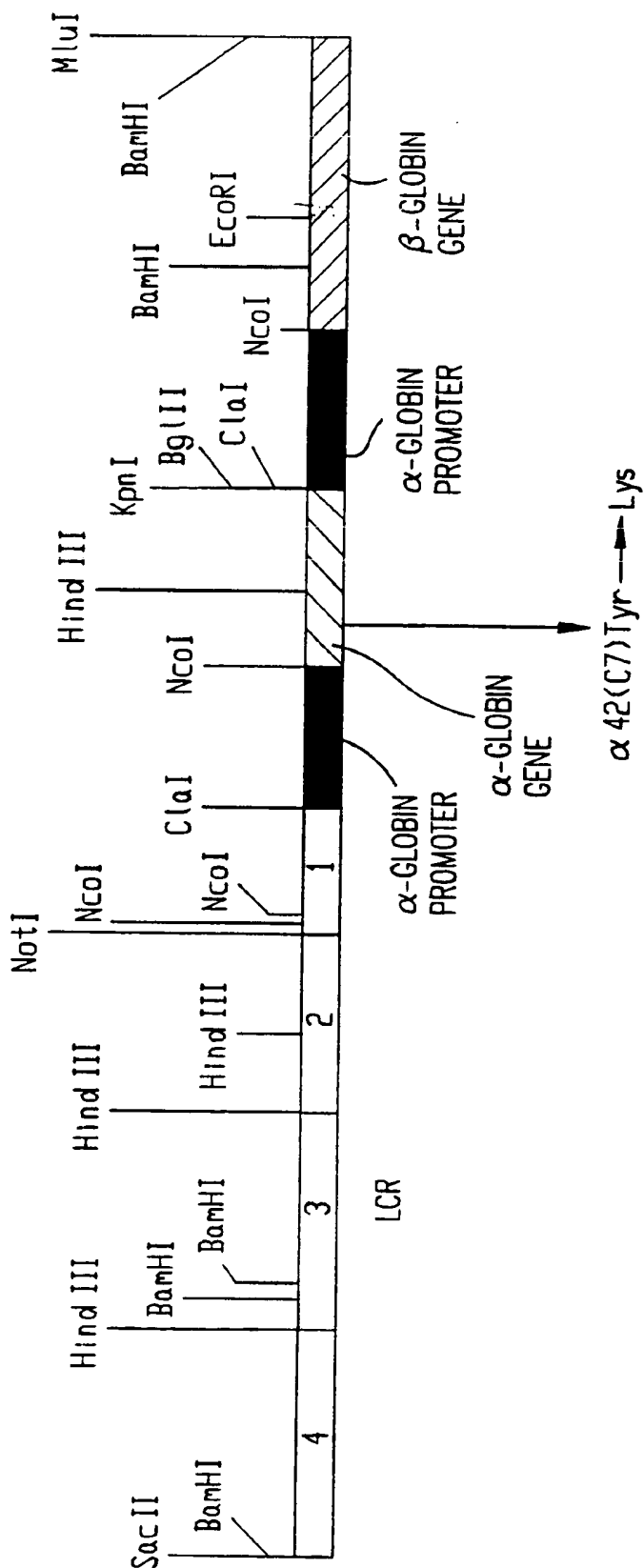


FIG.1W

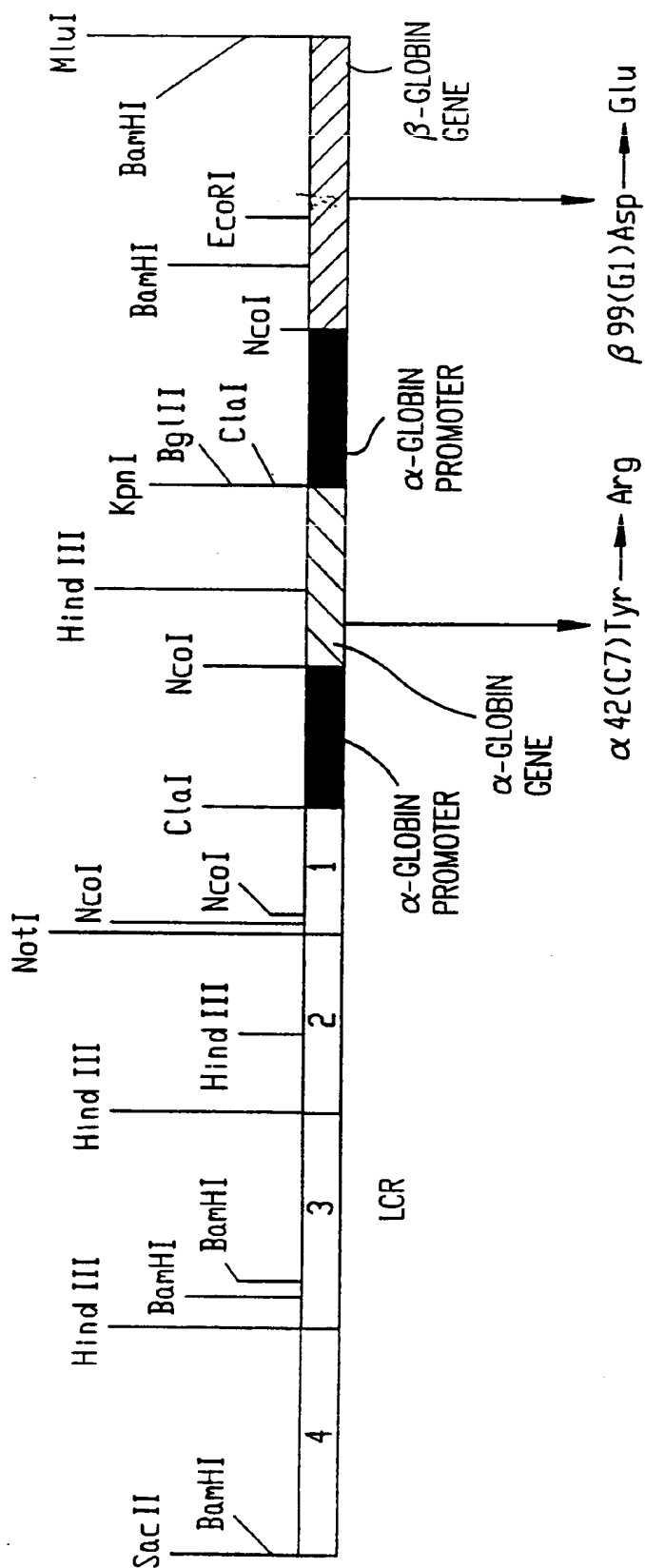


FIG. 1X

25/35

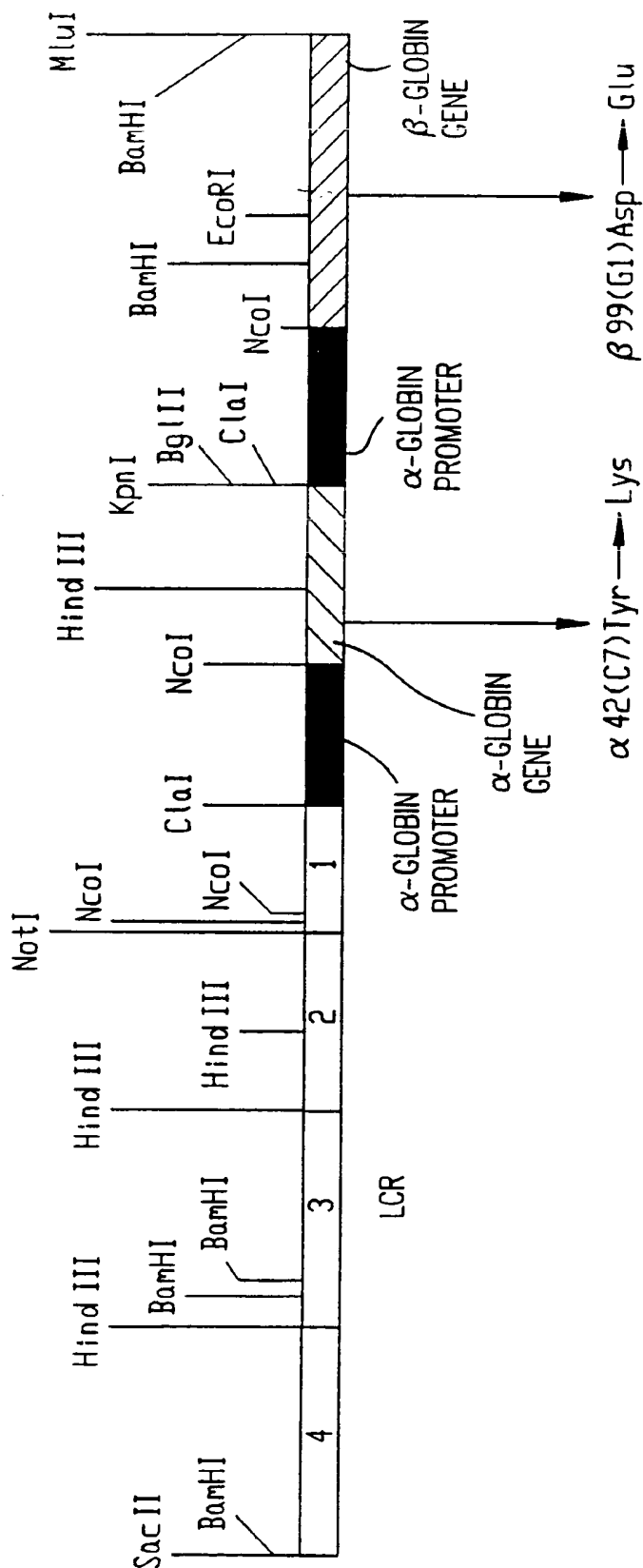


FIG.1Y

26/35



FIG. 2

SUBSTITUTE SHEET

27/35

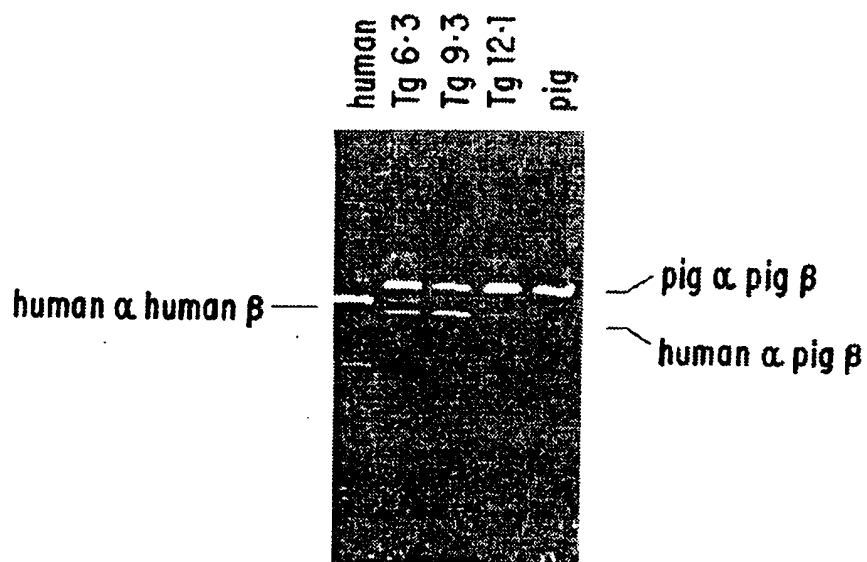


FIG. 3A

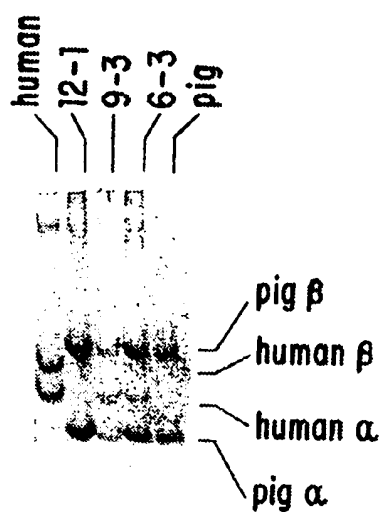


FIG. 3B

SUBSTITUTE SHEET

28/35

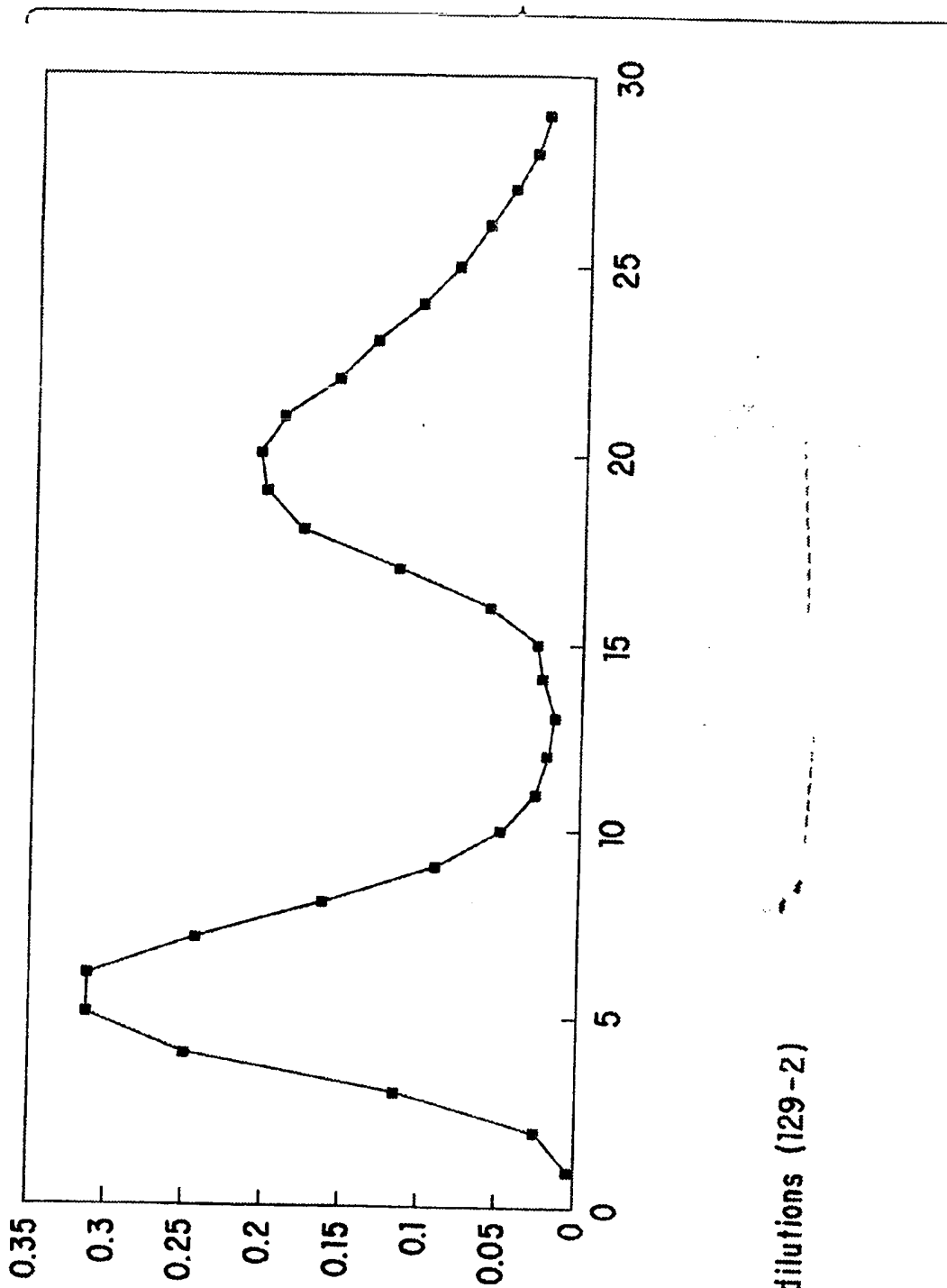


FIG. 4A

1:10 dilutions (129-2)

SUBSTITUTE SHEET

29/35

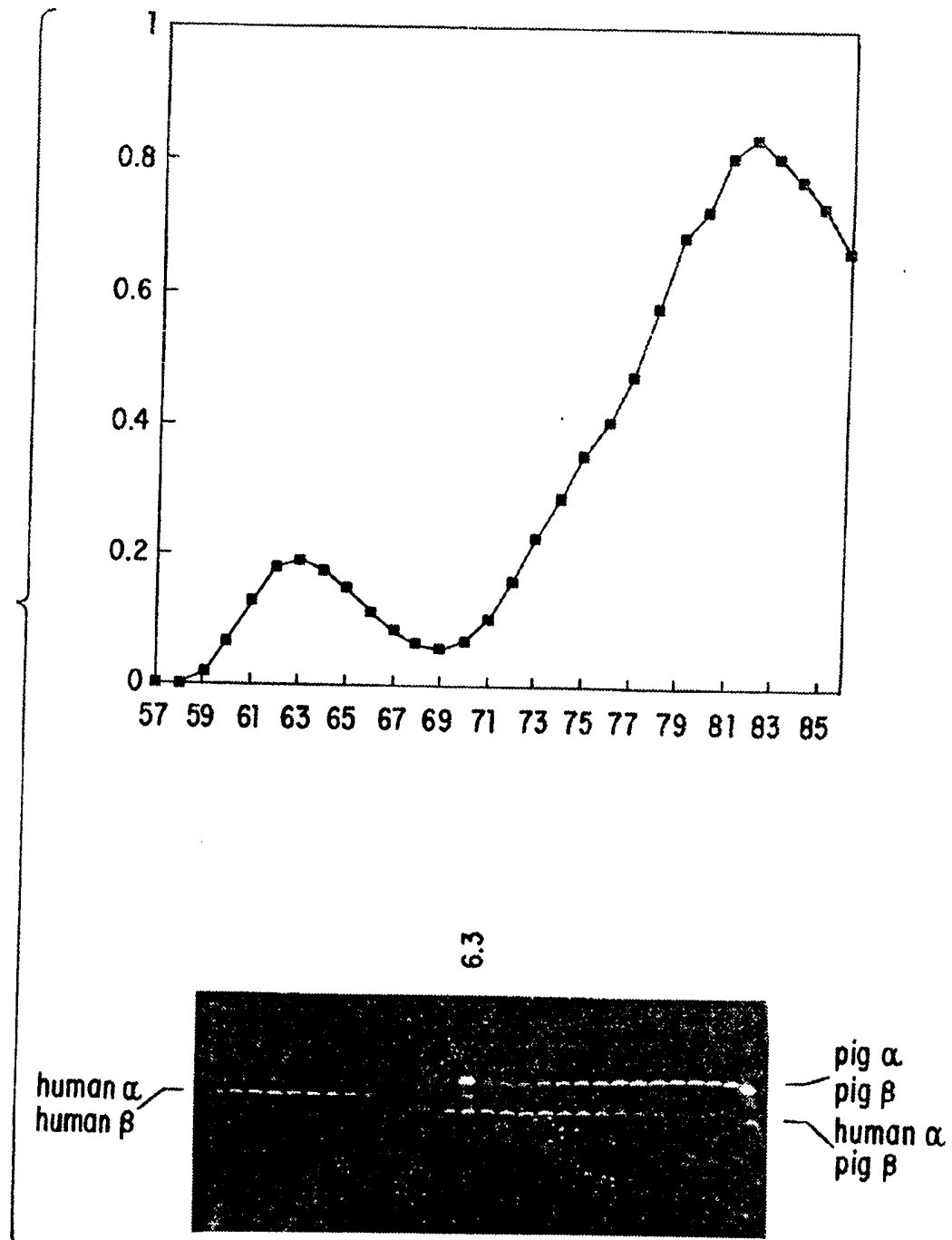


FIG. 4B SUBSTITUTE SHEET

30/35

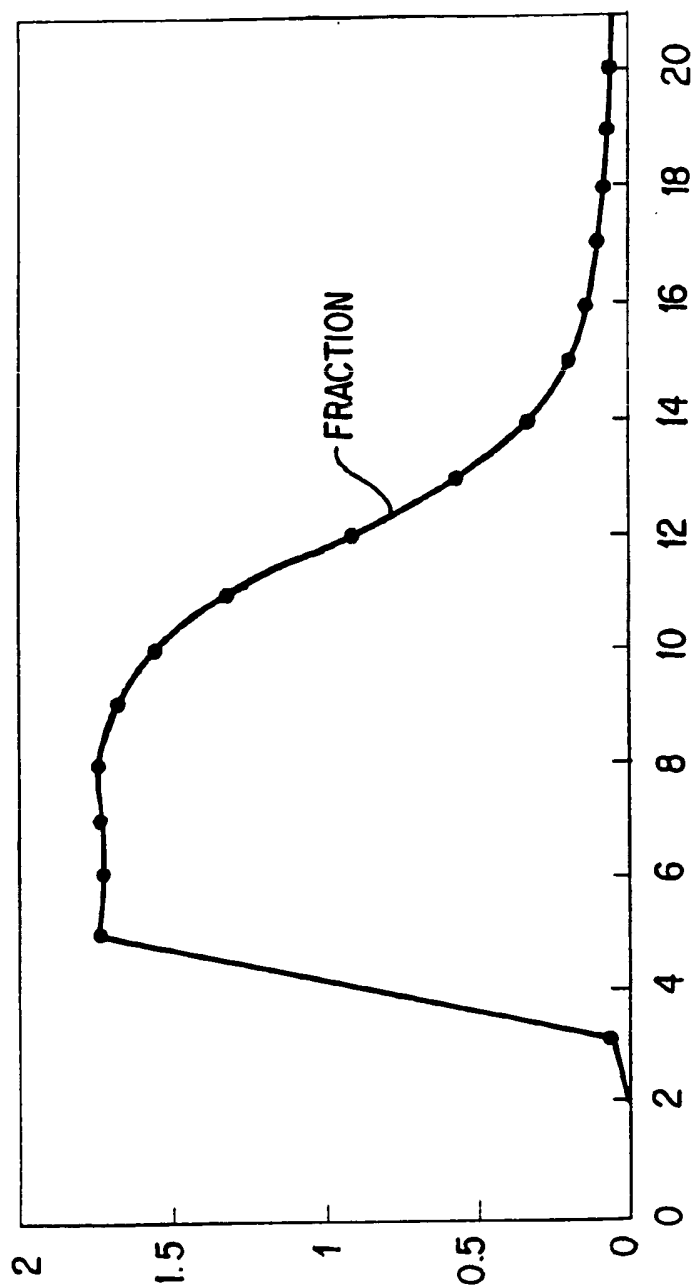


FIG. 4C

SUBSTITUTE SHEET

31/35

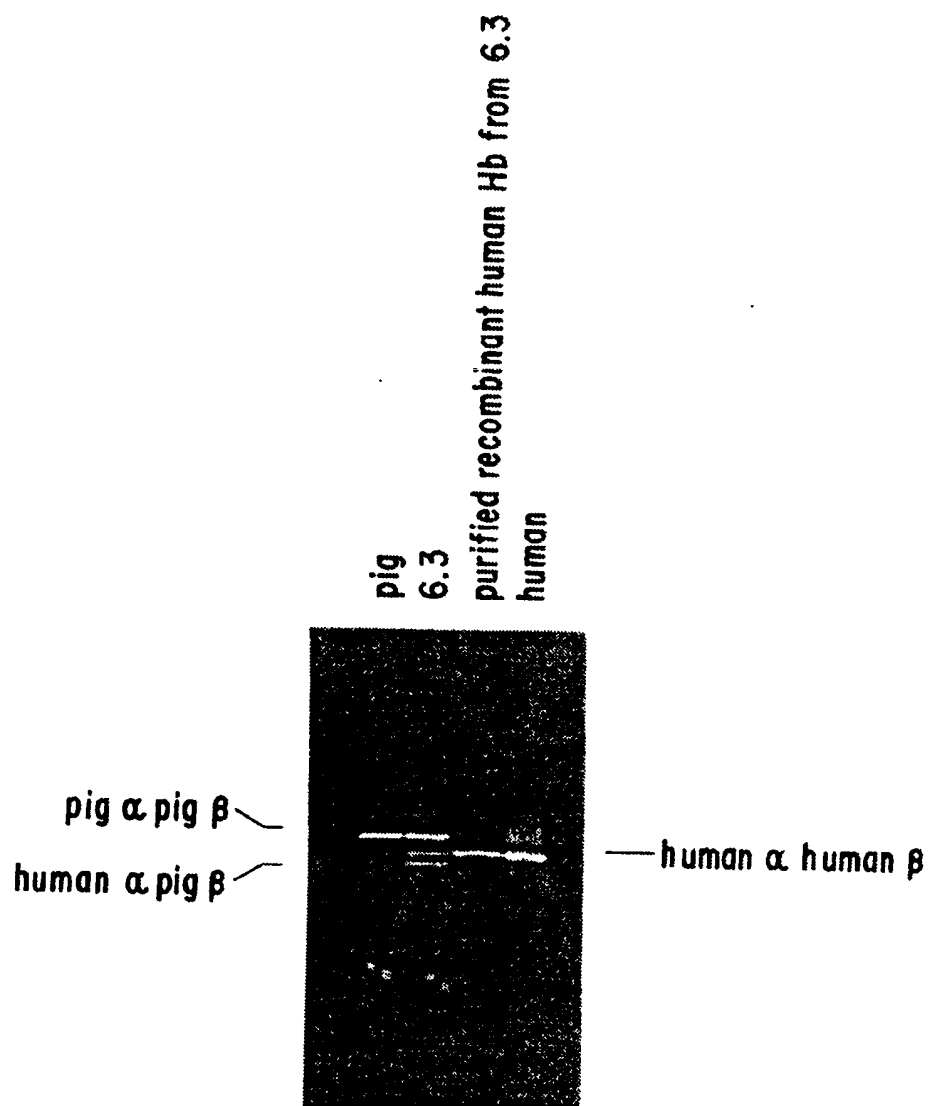


FIG. 4D

SUBSTITUTE SHEET

32/35

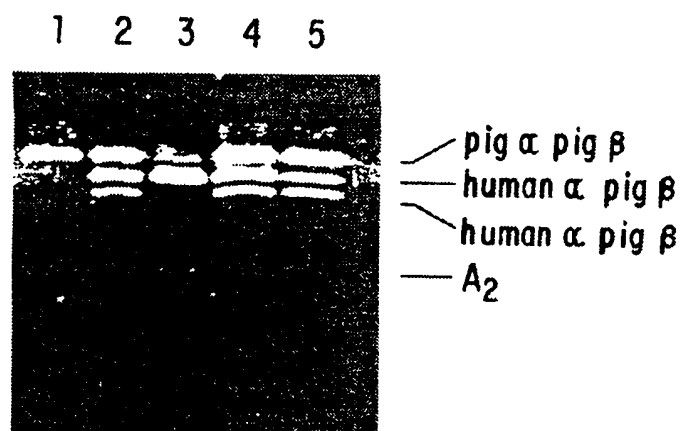


FIG. 5

SUBSTITUTE SHEET

33/35

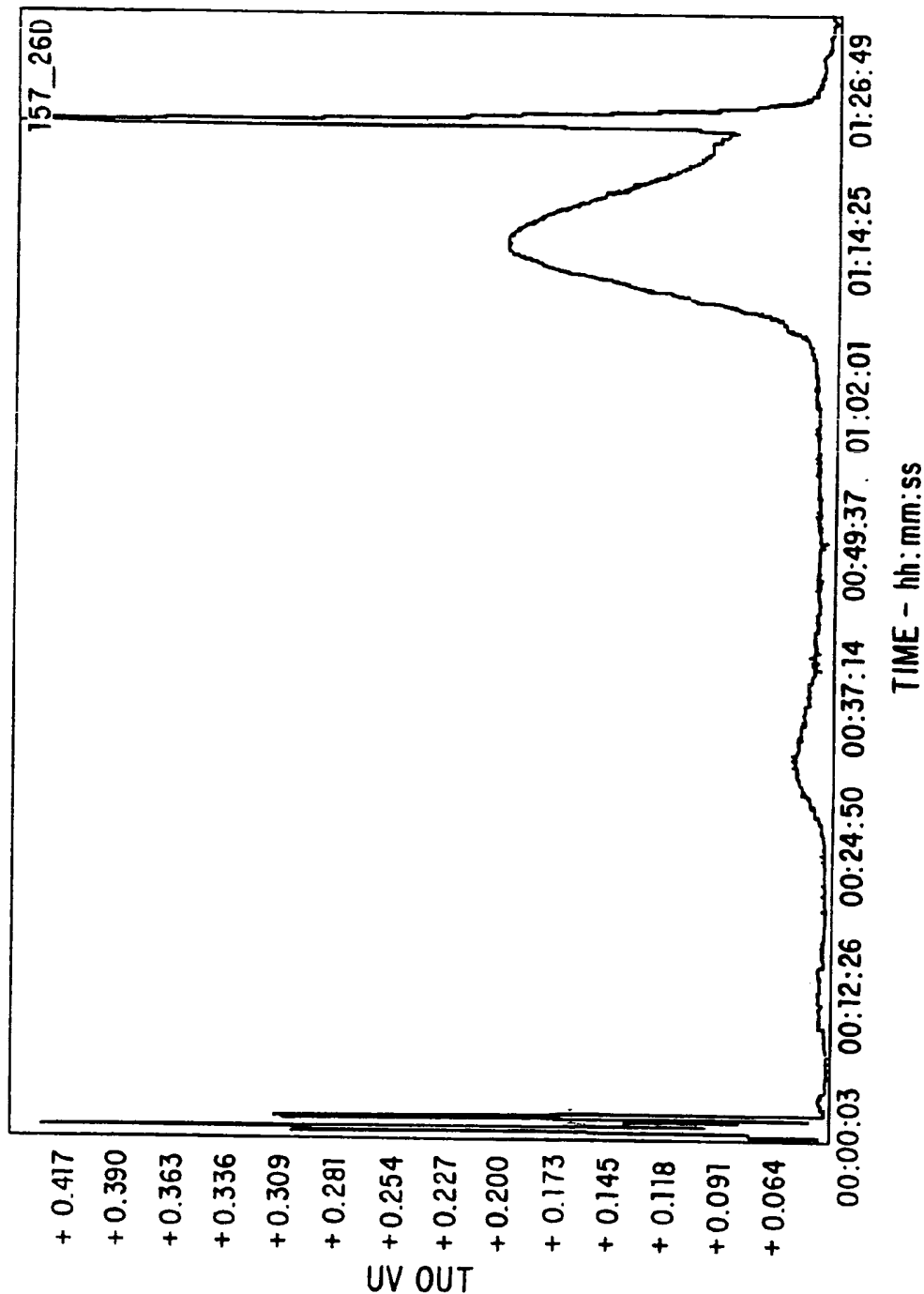


FIG. 6

34/35

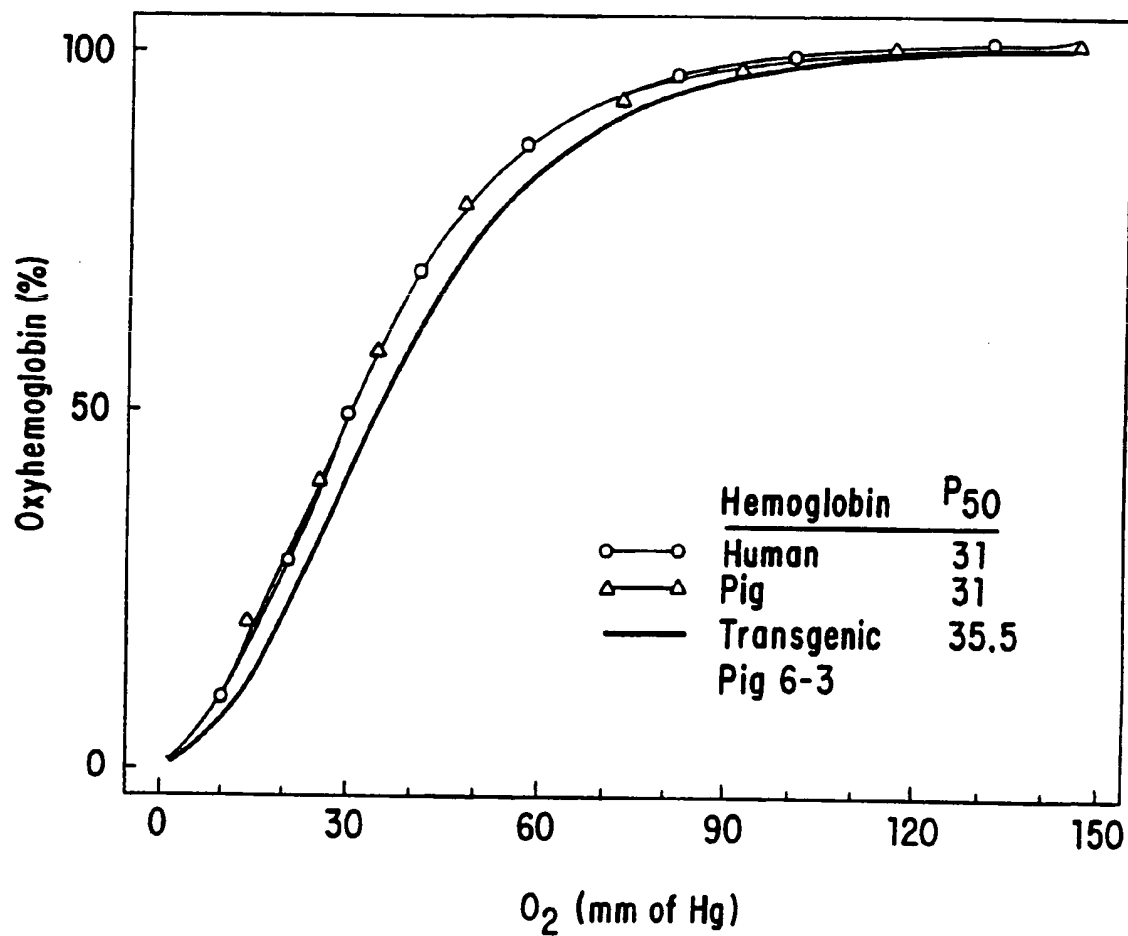


FIG. 7A

35/35

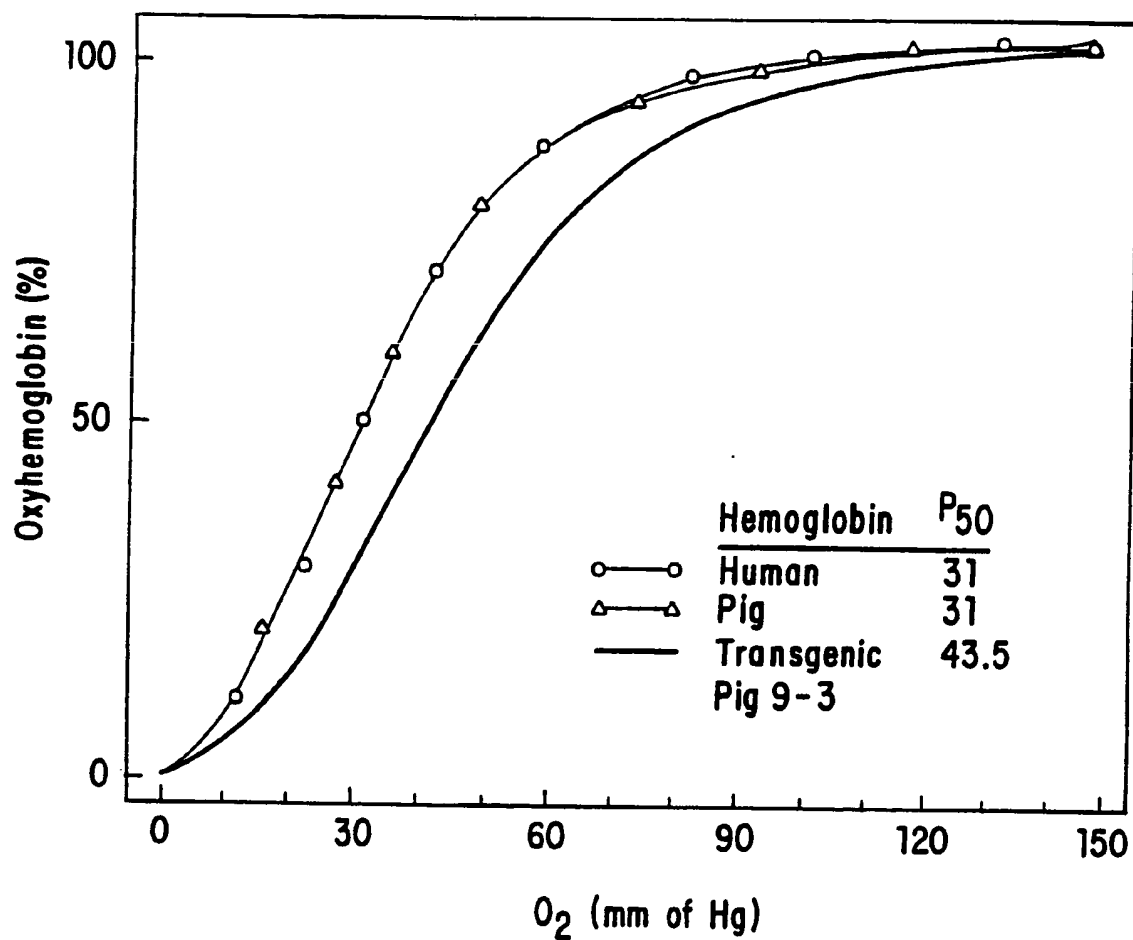


FIG. 7B

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05000

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12N 15/00; C12P 21/06; C07K 3/00, 7/00, 13/00
US CL : 800/2; 435/69.6; 530/416; 935/9, 60

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/2; 435/69.6; 530/416; 935/9, 60

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Science, volume 245, issued 01 September 1989, R.S. Behringer et al, "Synthesis of Functional Human Hemoglobin in Transgenic Mice", pages 971-973, entire document.	1-38
X	Nature, Volume 315, issued 20 June 1985, R.E. Hammer et al, "Production of Transgenic Rabbits, Sheep and Pigs by Microinjection", pages 680-683, entire document.	1-38
X	Proceedings of the National Academy of Science, Volume 81, issued September 1984, K. Young et al, "Stable Transfer and Expression of Exogenous Human Globin Genes in Human Erythroleukemia (K562) Cells", 5315-5319, entire document.	1-38
X	Nucleic Acids Research, Volume 76, issued 1981, A. Riggs, "Preparation of Blood Hemoglobins of Vertebrates", pages 5-29, Methods in Enzymology, entire document.	34-38

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 11 August 1992	Date of mailing of the international search report 20 AUG 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer DEBORAH CROUCH, PH.D. Telephone No. (703) 308-1126